Telomerase Recognizes G-Quadruplex and Linear DNA as Distinct Substrates†

Liana Oganesian, Mark E. Graham, Phillip J. Robinson, and Tracy M. Bryan*

Children's Medical Research Institute, 214 Hawkesbury Road, Westmead NSW 2145, Australia and University of Sydney, NSW 2006, Australia

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ABSTRACT: Telomeric DNA can assemble into a nonlinear, higher-order conformation known as a G-quadruplex. Here, we demonstrate by electrospray ionization mass spectrometry that the two repeat telomeric sequence d(TGGGGTTGGGGT) from *Tetrahymena thermophila* gives rise to a novel parallel four-stranded G-quadruplex in the presence of sodium. The G-quadruplex directly interacts with the catalytic subunit of *Tetrahymena* telomerase (TERT) with micromolar affinity, and the presence of telomerase RNA is not obligatory for this interaction. Both N- and C-terminal halves of TERT bind the G-quadruplex independently. This G-quadruplex is a robust substrate for both recombinant and cell extract-derived telomerase in vitro. Furthermore, the G-quadruplex weakens the affinity of wild-type telomerase for the incoming nucleotide (dTTP) and likely perturbs the nucleotide binding pocket of the enzyme. In agreement with this, a lysine to alanine substitution at amino acid 538 (K538A) within motif 1 of TERT dramatically reduces the ability of telomerase to extend G-quadruplex but not linear DNA. The K538A mutant retains binding affinity for the quadruplex. This suggests that telomerase undergoes changes in conformation in its active site to specifically accommodate binding and subsequent extension of G-quadruplex DNA. We propose that telomerase recognizes G-quadruplex DNA as a substrate that is distinct from linear DNA.

Telomeres are the structurally unique termini of linear chromosomes in eukaryotes, representing one of the signature motifs within the eukaryotic genome that is characteristically guanine-rich. Telomeres consist of a dispensable repetitive DNA sequence that prevents the loss of coding DNA that would otherwise occur with each cell division. The sequence of telomeric DNA varies between species but generally adheres to the consensus $T/A_{(1-4)}G_{(1-8)}$ (*1*). The telomeric repeat from the ciliate *Tetrahymena thermophila*, the organism that is the focus of the present study, is T_2G_4 (*2*). One of the distinctive features of the telomeric end is that the 5′−3′ strand protrudes as a single-stranded overhang, known as the G-overhang.

Chromosomal termini have been demonstrated to exist in nonlinear, higher order conformations. An example of such a conformation is the "T-loop" structure (*3*) observed in multiple species (*4*). This arises when the 3′ G-overhang invades the duplex region of the DNA upstream of the overhang, thereby forming a displacement loop (D-loop). An alternative higher order configuration has been observed in the ciliated protozoan *Stylonychia lemnae* (*5*, *6*) in the form of a G-quadruplex, a structural arrangement intrinsic to guanine-rich DNA. The G-quadruplex is an arrangement whereby four guanine residues are hydrogen bonded together in a cyclical fashion in Hoogsteen G−G base pairs (Figure 1A). The cavity that is formed at the core is the binding site for monovalent cations that serve to stabilize this structure (*7*). Synthetic DNA of telomeric sequence adopts a myriad of G-quadruplex conformations in vitro (reviewed in ref *8*). The number and orientation of DNA strands involved in the assembly of the G-quadruplex as well as the nature of the stabilizing cation are some of the factors contributing to this structural heterogeneity. Assembly of the G-quadruplex can be achieved either through a single DNA strand folding upon itself, thereby giving rise to an intramolecular G-quadruplex, or through the association of two or four DNA strands resulting in an intermolecular dimeric or tetrameric G-quadruplex, respectively. Both intra- and intermolecular G-quadruplex conformations can be subdivided into parallel and antiparallel categories, depending on the orientation of the DNA strands. Hybrid conformations have also been reported (*9*−*12*).

The telomeric G-overhang is the natural substrate for telomerase (*13*), a ribonucleoprotein enzyme that catalyzes de novo synthesis of telomeric DNA by reverse transcription of an integral RNA template (*14*). The telomerase reverse transcriptase (TERT)[1] protein, in association with the telomerase RNA (TER) bearing the template sequence, constitutes the core complex of telomerase, which is sufficient for catalytic activity of the human and *Tetrahymena* enzymes in vitro (*15*−*17*). Structurally, TERT is composed of a central

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* To whom correspondence should be addressed. Tel: + 61 2 9687 2800. Fax: + 61 2 9687 2120. E-mail: tbryan@cmri.usyd.edu.au.

[1] Abbreviations: BB, bromophenol blue; BSA, bovine serum albumin; HIV-1, human immunodeficiency virus-1; LDS, lithium dodecyl sulfate; MES, 2-(N-morpholino) ethane sulfonic acid; nanoESI-MS, nanoelectrospray ionization mass spectrometry; RT, reverse transcriptase; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TER, telomerase RNA; TERT, telomerase reverse transcriptase; UV, ultra violet; XC, xylene cyanol.

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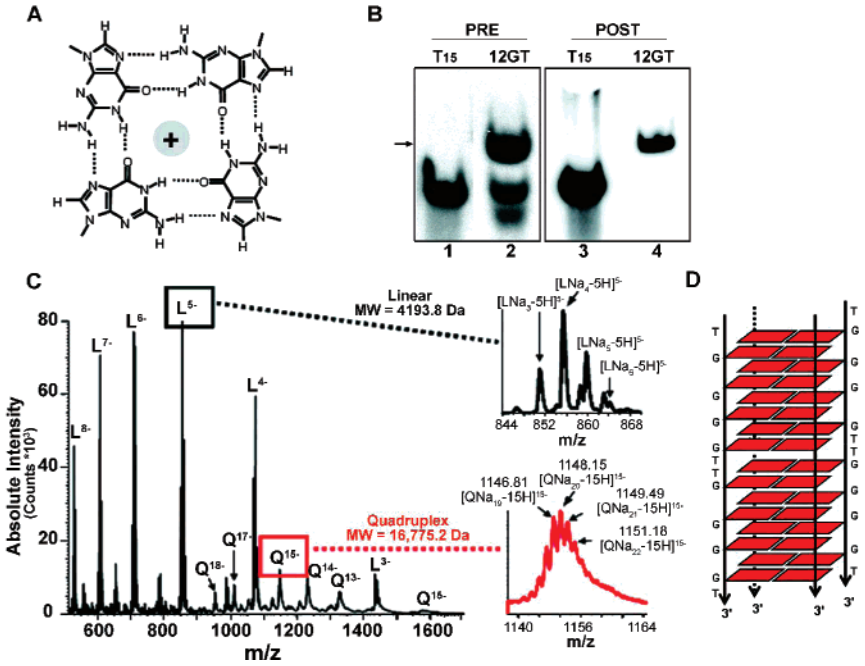


FIGURE 1: 12GT *Tetrahymena* telomeric sequence gives rise to a four-stranded intermolecular G-quadruplex. (A) Structure of a G-quartet. Hydrogen bonds between guanines and location of a centrally located monovalent cation are depicted. (B) Native gel electrophoresis of G-quadruplex conformations formed from 12GT *Tetrahymena* telomeric oligonucleotide (2 mM) in the presence of 100 mM NaCl pregel purification (lane 2; PRE). Gel purification of the intermolecular G-quadruplex species with the highest molecular weight (arrow) yields >98% pure G-quadruplex at 0.25 mM concentration postgel purification (lane 4; POST). Lanes 1 and 3, T_{15} molecular weight marker. Note that lanes 1 and 2 in the left panel of the figure are from the same gel; for clarity, intervening irrelevant lanes were excluded from the image. (C) NanoESI mass spectrum of a 20 μM solution of gel-purified Bio-12GT G-quadruplex in 10% propanol. DNA-sodium complexes were detected as linear (L) strands with approximately 2−6 sodium ions (upper inset, −5 charge state is shown) or as a quadruplex (Q) structure with approximately 15−25 sodium ions per quadruplex (lower inset, −15 charge state is shown). The ranges of the mass spectrum that were magnified and shown as insets are indicated by the black and red squares. (D) Proposed structure for the 12GT parallel four-stranded G-quadruplex.

reverse transcriptase (RT)-like domain that is flanked by amino (N)- and carboxy (C)-terminal extensions (see Figure 4A). The N-terminal extension includes the telomerase-specific motifs unique to TERT, while the central RT region bears the seven universally conserved domains shared by RT enzymes across phyla and harbors the active site of the enzyme (*18*, *19*).

Upregulated expression of telomerase is a hallmark of a majority (>85%) of cancer cells (*20*) and is necessary for their indefinite proliferative potential. Normal somatic cells, on the other hand, express the enzyme at very low or undetectable levels and therefore cease to proliferate after their telomeres reach a critically short length. Consequently, both telomerase and its substrate, the telomere, have become key targets for cancer therapy.

For some time, it has been widely believed that telomeric primers in G-quadruplex form, irrespective of particular conformation, are ineffective substrates for telomerase. This view stemmed from the observation that telomerase from the ciliate *Oxytricha nova* was unable to extend a potassium-stabilized intramolecular G-quadruplex in vitro (*21*). This provided an impetus for the development of G-quadruplex-stabilizing agents as in vivo telomerase inhibitors, which continues to be seen as an emerging avenue for cancer therapy (*22*).

We recently reported that not all G-quadruplex variants are refractory to telomerase extension, demonstrating that at least G-quadruplexes of the intermolecular parallel subclass efficiently prime ciliate telomerase in vitro (*23*). The current study extends our previous findings and presents a detailed structural characterization of a novel intermolecular G-quadruplex that arises from the two repeat *Tetrahymena* telomeric sequence $TG_4T_2G_4T$. The results offer new mechanistic insights into the extension of this nonlinear substrate by telomerase.

EXPERIMENTAL PROCEDURES

Oligonucleotides: Preparation and Labeling. DNA oligonucleotides used in this study (Table 1) were purchased from Sigma Genosys in desalted (T_{15}, 12GT, and Bio-12GT) or high-performance liquid chromatography (HPLC)-purified form (Bio-PBR and PBR48-Bio).

For radiolabeling, T_{15} and 12GT oligonucleotides (10 pmol each) were 5′-end-labeled with 25 pmol of [γ^{32}P]ATP (Perkin-Elmer, 6000 Ci/mmol) using 5−10 U of T4 polynucleotide kinase (Promega) for 30 min at 37 °C. PBR48-Bio (20 pmol) was 5′-end-labeled with ~23 pmol of [γ^{33}P]ATP (Perkin-Elmer, 3000 Ci/mmol) using 5−10 U of T4 polynucleotide kinase for 30 min at 37 °C. Radiolabeled DNA was purified on "Mini-Quick Spin columns for Oligos" (Roche) to eliminate unincorporated radioactive nucleotides.

Formation, Electrophoresis, and Purification of 12GT Four-Stranded G-Quadruplex. 12GT oligonucleotide of *Tetrahymena* telomeric sequence at 1−2.5 mM concentration was spiked with 2−10 × 10^6 cpm of radiolabeled oligonucleotide of the same sequence and heat denatured at 95 °C for 5 min in Telomerase buffer [50 mM Tris-HCl, pH 8.3, 1.25 mM $MgCl_2$, and 5 mM dithiothreitol (DTT)] and 100 mM sodium chloride (NaCl). After denaturation at 95

Table 1: Oligonucleotides Used in This Study

name	sequence (5′−3′)
12GT	TGGGGTTGGGGT
Bio-12GT	biotin-TGGGGTTGGGGT
Bio-PBR	biotin-AGCCACTATCGACTACGCGATCAT
PBR48-Bio	AGCCACTATCGACTACGCGATCATAGCCACTATCGACTACGCGATCAT-biotin
T_{15}	TTTTTTTTTTTTTTT
30AA	CCCCAACCCCAACCCCAACCCCAACCCCAA
48AA	CCCCAACCCCAACCCCAACCCCAACCCCAACCCCAACCCCAACCCCAA

°C in a heating block, the DNA was allowed to gradually reach 37 °C in the metal insert of the heating block and was incubated at this temperature for 2−3 days for optimal quadruplex formation. The quadruplex assembled DNA was then permitted to equilibrate to room temperature (RT) for 30−60 min, added to 6× native gel loading buffer [0.25% bromophenol blue (BB), 0.25% xylene cyanol (XC), and 30% glycerol], and electrophoresed on a nondenaturing 12% polyacrylamide gel for 4−5 h at 12 W at 25 °C. Both running buffer and gel mix contained the same constituents as those in which the DNA was incubated with the exception of DTT. Subsequently, the gel was exposed to BioMax Kodak sensitive film for 1−2 h to determine the location of the radiolabeled quadruplex with respect to the T_{15} molecular weight marker and BB and XC dyes. Comigration of the desired quadruplex band with the bulk of the unlabeled quadruplex DNA was confirmed by ultraviolet (UV) shadowing on a thin-layer chromatography plate (Merck). The band of lowest mobility was excised and crushed in TEN buffer (10 mM Tris-HCl pH 8.3, 1 mM EDTA, 250 mM NaCl) and incubated for 12−16 h at RT with rotation. The supernatant was filtered with a 0.22 μm filter, and the quadruplex DNA was precipitated with 2 volumes of absolute ethanol for 4−16 h at −20 °C. The precipitated product was resuspended in the original folding buffer. The final G-quadruplex concentration was determined by UV absorbance at 260 nm, taking into account that its molecular weight was four times that of its linear counterpart. An aliquot of the gel-purified 12GT four-stranded G-quadruplex was electrophoresed on a second native gel in the presence of 100 mM NaCl to determine its purity using ImageQuant TL software (Molecular Dynamics). The purity was invariably >98%. The remainder of the gel-purified four-stranded G-quadruplex was stored at 4 °C in the buffer in which it was originally assembled.

Biotin-conjugated 12GT (Bio-12GT) G-quadruplex used for DNA binding assays and mass spectral analysis was formed following the same procedure except that radiolabeled DNA was not included. The purity of unlabeled G-quadruplex was determined by measuring the efficiency of its extension by terminal transferase (TdT), a polymerase that extends the four-stranded G-quadruplex very poorly (*23*). The proportion of G-quadruplex extended (<8%) with respect to its denatured control reflected the level of its impurity.

Mass Spectral Analysis of G-Quadruplex. An aliquot (0.3 μL) of a 135 μM aqueous solution of gel-purified (see above) Bio-12GT quadruplex DNA in 50 mM Tris-HCl, pH 8.3, 1.25 mM $MgCl_2$, and 100 mM NaCl was loaded onto a microcolumn packed with POROS R3 material as described previously (*24*). The column was washed with 2 lots of 10 μL of water, eluted with 2 μL of 10% propanol (~20 μM DNA) into a nanoelectrospray (nanoESI) needle (~1 μm i.d., Proxeon Biosystems, Denmark), and immediately analyzed by nanoESI mass spectrometry (QSTAR XL, Applied Biosystems, United States). The sample was sprayed through the needle with −1200 V applied to the tip, and the declustering potential was at −30 V. Data were acquired in multiple channel acquisition mode for 30 min. The charge and the number of sodium ions in each mass peak were calculated manually.

Unfolding Assays (Complementary Strand Trap Method). Complementary strand "trap" assays were modified from previously published protocols (*23*, *25*). Briefly, ^{32}P-labeled gel-purified 12GT tetramer was incubated in the presence of an excess (10-fold strand excess or 40-fold molar excess) of complementary RNA [30AA; $(C_4A_2)_5$] or DNA [48AA; $(C_4A_2)_8$] at 37 °C. Aliquots of this hybridization reaction were removed at regular time intervals and loaded onto a native 12% polyacrylamide gel containing 100 mM NaCl. Electrophoresis was conducted at 12 W and attached to a circulating waterbath at 37 °C to maintain the hybridization temperature. The gel was dried, exposed to a PhosphorImager screen, and analyzed using ImageQuant software.

Plasmids. The construction of plasmids encoding a synthetic version of *T. thermophila* TERT gene with an N-terminal FLAG tag (pFLAG-TERT) or N-terminal T7 and His tags (pET-28a-TERT) has been described elsewhere (*26*, *27*). The mutant versions of the latter plasmid (K538A, K539A, L327A, K329A, C331A, P334A, R543A, I545A, Y623A, D624A, S778A, K849A, and S880A) were previously generated by site-directed mutagenesis and have been functionally characterized (*28*).

To construct a plasmid encoding FLAG-tagged amino acids 1−519 of TERT (plasmid FLAG-N519), this fragment was amplified using primers 5′-GTCGCAGGCGTCTGTTTGAATCAGTACTTTTCTGTC (forward) and 5′-CAGGATCTCGAGTCACAATTTTTCTTCCACTTTCTC (reverse), digested with ScaI and XhoI, and cloned into the ScaI and XhoI sites of FLAG-TERT, replacing full-length TERT with the fragment. To construct a plasmid encoding FLAG-tagged amino acids 520−1117 of TERT (plasmid FLAG-C598), this fragment was amplified using primers 5′-GATATACCATGGCTGACTACAAGGACGACGATGACAAGCATATGGGGGGATCCATCCCAGAAGATTCATTTCAG (forward) and 5′-CAGTGTCTTAATGTCCTGTATGAGGTTATTTAAATCTATGAATTCAATAGCCTCCAGTTCTTTTTTGTTCAGCTG (reverse), digested with NcoI and EcoRI, and cloned into the NcoI and EcoRI sites of FLAG-TERT, replacing full-length TERT with the fragment. pET-28 encoding amino acids 2−191 (TEN or GQ) was a gift from Thomas Cech and Steven Jacobs at the University of Colorado (Boulder, CO) (*29*). To construct a plasmid encoding FLAG-tagged amino acids

2−191, the TEN/GQ plasmid was digested with NdeI and BlpI and cloned into the NdeI and BlpI sites of FLAG-TERT, replacing full-length TERT with the fragment.

Preparation of in Vitro Reconstituted Tetrahymena Telomerase. Tetrahymena telomerase was reconstituted by translation of a plasmid encoding a synthetic TERT gene with an N-terminal FLAG tag or T7 and His tags (see above) in the presence of in vitro transcribed *Tetrahymena* TER (*26*) in rabbit reticulocyte lysates (TnT T7 Quick for PCR kit; Promega). Reconstitution reactions (400 μL) contained 320 μL of Quick Master Mix, 32 μL of [^{35}S]methionine (Perkin-Elmer; 1175 Ci/mmol; 10 μCi/μl), 8 μg of plasmid encoding the protein, and 50 nM TER. Translation was carried out at 30 °C for 60 min. Reconstituted enzyme was snap frozen in liquid nitrogen and stored at −80 °C or immunopurified. TERT protein fragments were translated in the same manner by using the relevant plasmids (see above).

Immunoprecipitation of in vitro translated telomerase was achieved using anti-FLAG M2 affinity (Sigma) or T7 tag antibody agarose (Novagen) beads, as described (*26*), with the exception that the buffers contained no Nonidet P-40 (which we have found to inhibit *Tetrahymena* telomerase activity). Telomerase-bound beads were resuspended in 1 volume of TMG buffer (10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, and 10% glycerol). Bead-bound telomerase was used for all direct primer extension assays.

For DNA binding assays and rNTPase and dNTPase assays, telomerase carrying the N-terminal FLAG tag was eluted off the beads by competition with 3 × FLAG peptide (Sigma). The telomerase bead slurry (200 μL) was washed once in wash buffer-0 (20 mM Tris-Acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, and 0.1 mM DTT). The beads were resuspended in 200 μL of peptide (0.75 mg/mL) in wash buffer-0 in a Protein LoBind tube (Eppendorf). Bovine serum albumin (BSA) was added (0.5 mg/mL) to prevent telomerase from sticking to the tube after elution. The bead slurry was rotated at 4 °C for 1 h and centrifuged at 1500*g* for 2 min at 4 °C, and the eluate was transferred to a fresh LoBind tube.

The yield of active enzyme was determined by dot blotting an aliquot of the enzyme along with a dilution series of *Tetrahymena* TER onto a Hybond N+membrane (Amersham Biosciences) and detecting the RNA in the telomerase complex by hybridizing the membrane with a radioactive probe complementary to *Tetrahymena* TER (5′-TATCAGCACTAGATTTTTGGGGTTGAATG-3′). The average final concentration of eluted FLAG-tagged telomerase was 5−9 nM.

For DNA binding assays involving His-tagged mutant K538A or wild-type telomerase, the enzyme was purified from a 125 μL translation reaction using TALON metal affinity resin (Clontech), eluted off the resin using imidazole, and desalted to remove imidazole, which inhibits *Tetrahymena* telomerase activity. TALON resin (300 μL per translation) was washed four times with IMAC buffer [50 mM $NaHPO_4$, pH 7.2, 150 mM potassium glutamate (KGlu), 5 mM $MgCl_2$, and 10% glycerol], centrifuged at 700*g* for 2 min at 4 °C between washes, and resuspended in 300 μL of IMAC buffer. The translation reaction was combined with 480 μL of IMAC buffer, added to the TALON resin, and incubated at 4 °C for 1 h with rotation. The telomerase-bound resin was washed four times with IMAC buffer, centrifuged at 700*g* for 2 min at 4 °C between washes, and resuspended in 360 μL of imidazole buffer (50 mM $NaHPO_4$, pH 7.2, 1 mM $MgCl_2$, 150 mM imidazole, 10% glycerol, and 0.5 mg/mL BSA). Elution was carried out at RT for 5 min with rotation followed by centrifugation at 700*g* for 2 min, after which the eluate was transferred to a Protein LoBind tube. The elution step was repeated, and the two eluate aliquots were combined. Four Micro Bio-Spin 6 chromatography columns (BioRad) per eluate were used for desalting purposes. The columns were prepared by centrifugation at 1000*g* for 2 min at 4 °C, the buffer was removed, and the columns were washed twice with wash buffer-1 (20 mM Tris-Acetate, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 0.1 mM DTT, and 1 mM KGlu). To block the columns, 600 μL of wash buffer-1 with 0.5 mg/mL of BSA was applied to each column and incubated for 30 min at RT. The columns were then centrifuged twice at 1000*g* for 2 min until completely dry to avoid unnecessary eluate dilution. The eluate was applied to each column in two 90 μL batches, with centrifugation for 4 min at 1000*g* between applications. Desalted eluate from each of the four columns was pooled, resulting in a total volume of ∼720 μL of active telomerase per translation reaction.

Preparation of Tetrahymena Extracts. T. thermophila strains B2086.1 and CU428.2 were gifts from Donna Cassidy-Hanley (Cornell University, Ithaca, NY). B2086.1 cells were grown vegetatively in 20 mg/mL proteose peptone (Difco) and 100 μM $FeCl_3$ at 30 °C with shaking at 100 rpm to a cell density of 1 × 10^5 cells/mL and then collected by centrifugation for 10 min at 1000*g*. To initiate starvation, cells grown to a density of 1 × 10^5 cells/mL were collected by centrifugation, washed twice in Dryl's medium (1.7 mM trisodium citrate, 1.2 mM NaH_2PO_4, 1.3 mM Na_2HPO_4, and 2 mM $CaCl_2$), and incubated at a density of 3 × 10^5 cells/mL in Dryl's medium at 30 °C without shaking for at least 12 h. Starved cells were induced to conjugate by mixing equal numbers of the two mating types in starvation medium without shaking, and cells were harvested 5 h after initiation of mating (at which point 95% of cells were paired). S100 extracts were made from both vegetative and mated cells as described previously (*30*). The extracts were partially purified on a 2 mL DEAE agarose (Bio-Rad) column as described (*27*). Fractions containing active telomerase were pooled, dialyzed for 3 h into TMG + 5 mM β-mercaptoethanol at 4 °C, flash frozen, and stored at −80 °C. The concentration of telomerase was estimated by electrophoresis on native agarose-acrylamide gels (*31*), transfer to Hybond N+ (Amersham Biosciences) and hybridization of the membrane with a radioactive probe complementary to *Tetrahymena* telomerase RNA (5′-TATCAGCACTAGATTTTTGGGGTTGAATG-3′). The final concentration of telomerase was ∼50 pM.

Telomerase Activity Assays. Assays were performed using in vitro reconstituted and immunopurified *Tetrahymena* telomerase or partially purified *Tetrahymena* extracts (see above). Various concentrations of 12GT DNA primer (see text) in G-quadruplex, denatured, or linear conformations were used as substrates. For the denatured samples, G-quadruplex was formed (in Telomerase buffer containing 100 mM NaCl) and gel-purified as outlined above, then heat denatured at 95 °C for 5 min, and snap cooled on ice immediately prior to the start of the reaction. Linear 12GT was resuspended in Telomerase buffer without NaCl. For

recombinant telomerase, the DNA was incubated at 25 °C for 15−30 min in the presence of Telomerase buffer, 100 μM dTTP (Roche), 10 μM [α^{32}P]dGTP at 80 Ci/mmol {0.4 μL of nonradioactive dGTP at 487 μM and 1.6 μL of [α^{32}P]dGTP (Perkin-Elmer; 10 μCi/μL, 3000 Ci/mmol)}, and 10 μL of 1:1 slurry of telomerase beads in a 20 μL reaction. For extract-derived telomerase, the DNA was incubated at 25 °C for 60 min in the presence of Telomerase buffer, 100 μM dTTP (Roche), 1.25 μM [α^{32}P]dGTP at 800 Ci/mmol {0.2 μL of nonradioactive dGTP at 92 μM and 2 μL of [α^{32}P]dGTP (Perkin-Elmer; 10 μCi/μL, 3000 Ci/mmol)}, and 10 μL of extract in a 20 μL reaction. Reactions also included 100 mM NaCl (for the G-quadruplex and denatured primer) or no salt (for the linear primer). The reaction was terminated by adding 80 μL of TES (50 mM Tris-HCl, pH 8.3, 20 mM EDTA, and 0.2% SDS). A ^{32}P-labeled 100-mer oligonucleotide (5000 cpm) was added as a recovery and loading control. The reaction products were phenol/chloroform extracted and ethanol precipitated for 1−16 h at −20 °C. The pellet was washed once in 70% ethanol, air-dried, resuspended in 5 μL of formamide loading dye [90% deionized formamide, 0.1% BB, and 0.1% XC in TBE (89 mM Tris, 89 mM Borate, and 2 mM EDTA) buffer], and heat-denatured at 95 °C for 5 min, and half of each reaction was electrophoresed on a denaturing 12% polyacrylamide/8 M Urea sequencing type gel for 1.5 h at 80 W. The gel was dried for 1 h at 80 °C, exposed to a PhosphorImager screen (Molecular Dynamics), and analyzed using ImageQuant TL software.

K_m measurements were carried out by performing the assays within the linear phase of the reaction (15 min of incubation). The total intensity of extension products at each substrate concentration was normalized against the intensity of the ^{32}P-labeled 100-mer recovery and loading control. The resulting values were expressed as a percentage of the reaction with maximal activity and plotted against substrate concentrations [S]. This was fitted to the Michaelis−Menten kinetics equation, $y = (V_{max} \times [S])/(K_m + [S])$, to yield the affinity constant K_m.

For initiation reactions, the reaction was carried out for 15 min at 25 °C. G-quadruplex, denatured, and linear primer at 12 μM concentration were incubated in the presence of Telomerase buffer with (for G-quadruplex and denatured primer) or without (for linear primer) 100 mM NaCl, 1.3 μM [α^{32}P]dTTP {0.2 μL of nonradioactive dTTP at 92 μM and 2.5 μL of [α^{32}P]dTTP (Perkin-Elmer; 10 μCi/μL, 3000 Ci/mmol)}, 100 μM ddGTP (Roche), and 10 μL of 1:1 slurry of telomerase beads in a 20 μL reaction. The reactions were terminated and processed as described above.

The efficiency (total product formation) with which wild-type telomerase or its mutant counterparts extended 12GT four-stranded G-quadruplex was quantitated using ImageQuant software, normalized against the intensity of the ^{32}P-labeled 100-mer recovery and loading control, and expressed as a percentage of extension of the denatured form of this quadruplex. The level of quadruplex extension relative to that of its denatured form was compared between the wild-type enzyme and the single-point mutants.

DNA Binding Assays. DNA binding studies were conducted by immobilizing biotinylated 12GT tetrameric G-quadruplex on UltraLink Immobilized NeutrAvidin Protein Plus beads (Pierce Biotechnology) in the presence of radiolabeled recombinant *Tetrahymena* telomerase. Nonradioactive biotinylated 12GT oligonucleotide was assembled into a four-stranded G-quadruplex and gel purified as described above. The quadruplex (8 μL) or its denatured counterpart (heat denatured at 95 °C for 5 min and then snap cooled on ice) at 0.2−20.0 μM concentrations or Bio-PBR nontelomeric DNA (see Table 1) at 2.5 μM were incubated with 12 μL of ^{35}S-labeled recombinant telomerase eluate (see above) in Telomerase buffer (see above), 100 mM NaCl, and 10% glycerol in the presence of 2500 cpm of ^{33}P-labeled PBR48-Bio (recovery and loading control; see Table 1) at 25 °C for 15 min. NeutrAvidin beads (10 μL of beads per reaction) were washed four times with Telomerase buffer plus 100 mM NaCl and 10% glycerol and centrifuged at 1500*g* for 2 min at 4 °C between washes. The beads were blocked twice for 15 min or overnight at 4 °C in the same buffer with the addition of 0.75 mg/mL BSA, 0.15 mg/mL glycogen, and 0.15 mg/mL yeast RNA. Blocked beads were resuspended in 1 volume of blocking buffer. Blocked bead slurry (20 μL) was added to the DNA-telomerase reaction mix, and the samples were incubated at 4 °C for 15 min with rotation. The DNA-protein bound beads were washed four times with Telomerase buffer, 300 mM NaCl, and 10% glycerol wash buffer and centrifuged at 1500*g* for 2 min at 4 °C between washes. The beads were resuspended in 2 volumes of the wash buffer. Half of each reaction (15 μL) was added to NuPage 4 × lithium dodecyl sulfate (LDS) sample buffer (5 μL) (Invitrogen), heated at 95 °C for 5 min, and electrophoresed on 4−12% NuPage gels in MES [2-(N-morpholino)ethane sulfonic acid] buffer (Invitrogen) for 35 min at 200 V. The gels were fixed for 20−30 min in 25% isopropanol/10% acetic acid, dried at 80 °C for 1 h, exposed to a PhosphorImager screen, and analyzed using ImageQuant TL software. The total intensity of the protein captured at each substrate concentration was normalized against the intensity of the ^{33}P-labeled PBR48-Bio loading control. The signal from the reaction containing no DNA, representing background binding to the beads, was subtracted from all others. The resulting values were expressed as a percentage of the reaction with maximal protein pull-down and plotted against substrate concentrations [S]. This was fitted to the following equation, $y = (B_{max} \times [S])/(K_d + [S])$, where B_{max} is the maximal level of binding, to yield the dissociation constant K_d.

RESULTS

Telomeric Sequence 12GT from Tetrahymena Gives Rise to a Novel Parallel Four-Stranded G-Quadruplex. The solution structure of 12GT *Tetrahymena* telomeric sequence has been determined to be an antiparallel dimer in the presence of 100 mM NaCl (*32*). In our attempt to form this structure, we observed that this sequence also gives rise to an additional slower-migrating conformation detected on a nondenaturing gel in the presence of 100 mM Na^+ (Figure 1B, lane 2; arrow). This higher molecular weight DNA species, once gel purified, completely conserves its original conformation and exhibits >98% purity postgel purification (Figure 1B; lane 4). We have previously shown by circular dichroism spectroscopy that this DNA species is a parallel G-quadruplex. Moreover, UV cross-linking analysis was strongly suggestive of a four-stranded structure (*23*). Here, we have measured the exact molecular weight of this

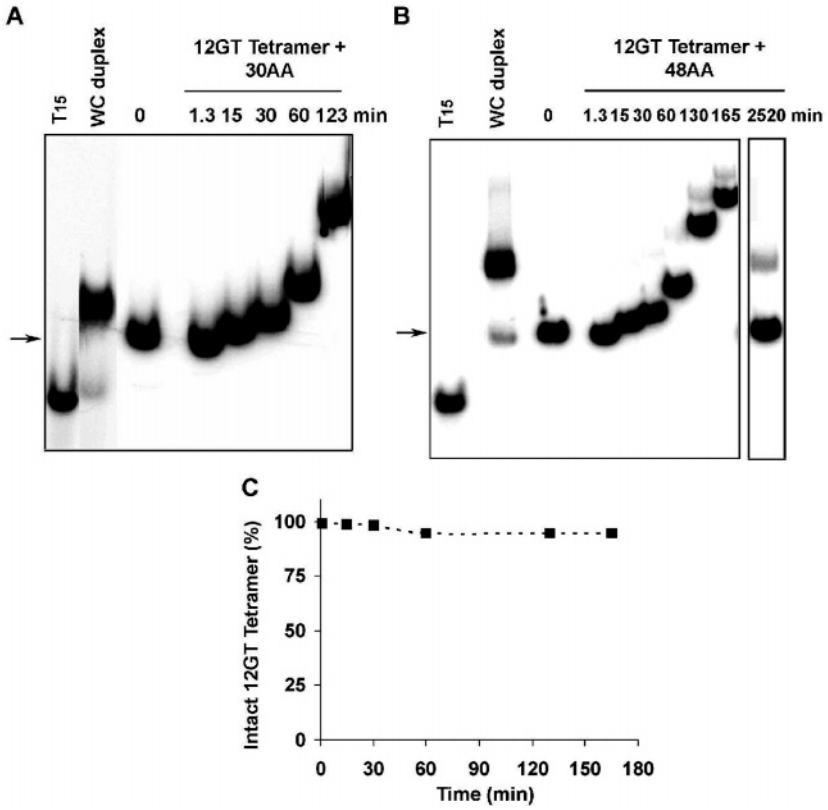


FIGURE 2: Complementary strand trap assays for determining the unfolding rate of 12GT tetrameric G-quadruplex at 37 °C. ^{32}P-labeled 12GT tetramer was incubated with 40-fold molar excess of complementary single-stranded RNA [30AA; $(C_4A_2)_5$ at 700 μM] (A) or DNA [48AA; $(C_4A_2)_8$ at 560 μM] (B) at 37 °C, and at the indicated time intervals, aliquots of each hybridization mix were loaded onto a native 12% polyacrylamide gel containing 100 mM NaCl. T15, molecular weight marker; WC duplex, preannealed Watson−Crick duplex (12GT tetramer was heat-denatured at 95 °C for 5 min in the presence of excess complementary strand and then incubated at 37 °C for 30 min to allow for annealing). The arrow indicates the migration of the intact 12GT tetramer at 0 min of hybridization. Note that later time points have less time to migrate into the gel, with the exception of the right most panel in B, where the hybridization mix was preincubated for 2520 min (42 h) prior to loading onto the gel. Plot C shows the quantitation of the gel in B. Each gel is representative of two independent experiments.

G-quadruplex using nanoelectrospray ionization mass spectrometry (nanoESI-MS) in negative ionization mode (Figure 1C). The G-quadruplex (molecular mass = 16775.2 Da without metal ions) was detected in a range of charge states (−11 to −18). By calculating the mass of the neutral complex, it was determined that the DNA had between 15 and 25 sodium ions per quadruplex. Quadruplexes with 19−21 sodiums were most abundant (Figure 1C, lower inset). The dissociated (linear) form of the quadruplex was also detected in complex with 2−6 sodiums (Figure 1C, upper inset), presumably because of the reduced stability of the quadruplex in 10% propanol in the absence of NaCl; this was necessary for ESI-MS (see the Experimental Procedures). Detection of the G-quadruplex under such unfavorable buffer conditions is a testament to its high stability. Overall, the quadruplex displayed a molecular weight four times that of its linear counterpart (Figure 1C), confirming that it is a parallel four-stranded G-quadruplex (*23*; and Figure 1D), a conformation hitherto not reported for this sequence.

The Four-Stranded Parallel G-Quadruplex Is Highly Stable. We have previously reported that the 12GT four-stranded G-quadruplex has an undetectable unfolding rate at 25 °C (*23*). Here, we sought to assess the stability of this structure at a higher temperature, 37 °C, likely to be more conducive to unfolding. This was achieved by employing the complementary strand "trap" assay whereby the gel-purified 12GT G-quadruplex was incubated in the presence of a large excess of RNA or DNA of complementary sequence that act to "trap" the spontaneously dissociated quadruplex in a Watson−Crick (WC) duplex conformation. The products of hybridization were analyzed over time by nondenaturing electrophoresis. The rate of WC duplex formation was indicative of the rate of G-quadruplex unfolding. The 12GT tetramer displayed little or no unfolding within the first 2 h of hybridization with complementary RNA or DNA (Figure 2); even after 42 h of hybridization, <10% of the quadruplex contributed to WC duplex formation (Figure 2B,C). Hence, the 12GT four-stranded G-quadruplex is exceptionally stable.

Telomerase Binds and Extends the Four-Stranded Parallel G-Quadruplex. Having established the parallel four-stranded conformation of the G-quadruplex and its stability, telomerase activity assays were performed to determine the affinity of telomerase for this quadruplex. We have previously demonstrated that this Na^+-stabilized 12GT intermolecular G-quadruplex can be extended by recombinant *Tetrahymena* telomerase, but the affinity of the interaction was not determined (*23*). Figure 3A reinforces our previous observation; there is a robust extension of the tetramer at all concentrations tested, albeit to a lesser extent than the controls, denatured, and linear 12GT. The former is derived by forced heat denaturation of the G-quadruplex and hence contains salt and DNA strand concentrations identical to the

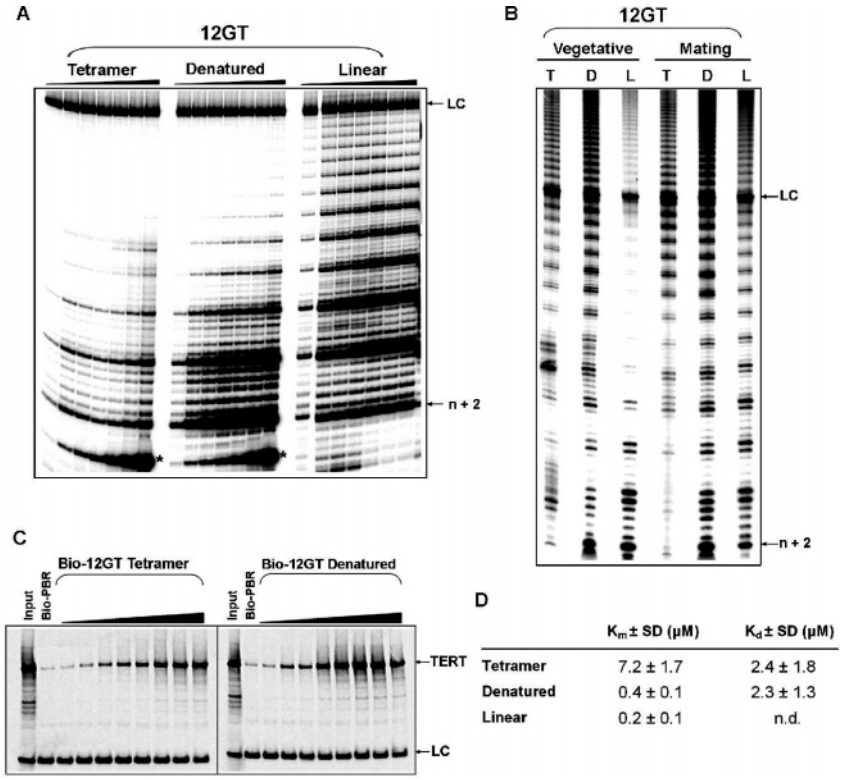


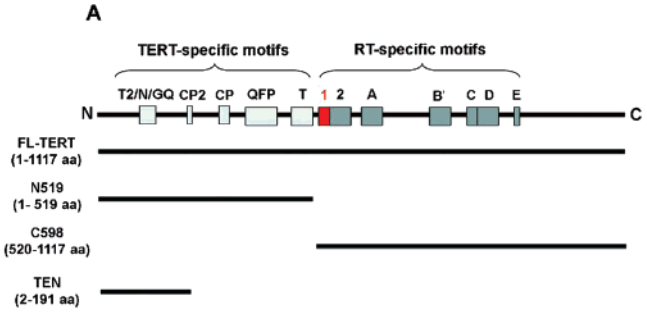
FIGURE 3: Characterization of telomerase interactions with the 12GT four-stranded G-quadruplex. (A) Direct primer extension assay of ^{32}P end-labeled gel-purified 12GT tetramer and its denatured (+NaCl) and linear (−NaCl) counterparts at 0.05, 0.25, 0.5, 1, 2.5, 5, and 10 μM concentrations using in vitro reconstituted recombinant immunopurified *Tetrahymena* telomerase. "n+2" indicates the first two nucleotides incorporated into the primer, "LC" is a ^{32}P-labeled 100-mer DNA oligonucleotide used as a recovery and loading control, and * represents the unextended 5′ ^{32}P end-labeled gel-purified G-quadruplex. (B) Direct primer extension assay of ^{32}P end-labeled gel-purified 12GT tetramer (T) and its denatured (D; +NaCl) and linear (L; −NaCl) counterparts at 10 μM concentration using partially purified native telomerase derived from vegetative and mating *Tetrahymena* extracts. "n+2" indicates the first two nucleotides incorporated into the primer, and "LC" is a ^{32}P-labeled 100-mer DNA oligonucleotide used as a recovery and loading control. (C) DNA binding assay to directly measure the affinity of telomerase for G-quadruplex. Biotinylated gel-purified 12GT tetramer and its denatured counterpart at 0, 0.2, 0.5, 1, 2.5, 10, 15, and 20 μM concentrations or nontelomeric control (Bio-PBR) at 2.5 μM were incubated with ^{35}S-methionine-labeled full-length recombinant telomerase (TERT + RNA) and recovered on NeutrAvidin beads. Note that the concentrations of the G-quadruplex are calculated based on the molecular weight of the four-stranded form of 12GT oligonucleotide. "Input" represents 50% of starting material in the bound lanes. "LC" is a ^{33}P-labeled PBR48-Bio oligonucleotide used as a loading and recovery control. (D) Extension activity and binding were quantitated from gels such as those shown in A and C; the derived affinity constants K_m (from the activity assays) and K_d (from the binding assays) are shown as the means ± standard deviations of 3−4 independent experiments. ND, not determined.

G-quadruplex sample. It is a necessary control since NaCl substantially reduces the repeat addition processivity of telomerase. The "linear" control refers to the DNA of the same sequence and molar concentration but in the absence of NaCl.

The extension of G-quadruplex DNA is not unique to the recombinant system in that the quadruplex efficiently acts as a substrate for native telomerase derived from both vegetatively growing and conjugating *Tetrahymena* cells (Figure 3B). The quadruplex extension by the recombinant enzyme was not dependent on ribo- or deoxyribonucleotide hydrolysis (Supporting Information, Figure 1). Thus, telomerase does not require an energy source to fuel the extension of a G-quadruplex substrate, although it remains possible that a level of hydrolysis is occurring that is below the detection limit of our assay.

The Michaelis−Menten affinity constant (K_m) derived from the telomerase activity assays (Figure 3A) revealed a bias of telomerase for an unstructured substrate. Accordingly, K_m for denatured and linear forms of the DNA was more than an order of magnitude lower than that for the quadruplex (Figure 3D). Hence, while telomerase exhibited higher affinity for DNA in denatured non-G-quadruplex form, it nonetheless had low micromolar affinity for the parallel four-stranded G-quadruplex.

To establish whether the extension of this quadruplex is facilitated through a direct interaction with telomerase, a DNA binding assay developed in our lab (*23*; S. Finger and T. Bryan, submitted for publication) was utilized, wherein gel-purified biotinylated 12GT tetramer was incubated with ^{35}S-labeled immunopurified *Tetrahymena* telomerase (TERT + RNA). The G-quadruplex/telomerase complex was then captured using Neutravidin-coated beads, and the amount of protein recovered was visualized on an sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The biotinylated 12GT tetramer bound to TERT with a binding affinity (K_d) comparable to that of its denatured counterpart (Figure 3C,D). Note that linear (no salt) controls were not included since in the absence of salt the affinity of the interaction is considerably higher (S. Finger and T. Bryan, submitted for publication); hence, the denatured form of the quadruplex is the more appropriate unstructured control. The observed interaction was specific at all concentrations tested since the level of protein pull-down was always higher than



B

$K_d \pm$ SD (μM)

	+ RNA	- RNA	
FL-TERT	2.4 ± 1.8	> 20*	T
	2.3 ± 1.3	1.3 ± 0.8	D
N519	3.3 ± 0.1	2.1 ± 0.8	T
	2.4 ± 0.4	1.1 ± 0.6	D
C598	2.7 ± 0.8	3.5 ± 2.9	T
	0.7 ± 0.3	0.9 ± 0.7	D
TEN	bg	bg	T
	n.d.	n.d.	D

FIGURE 4: Both N- and C-terminal regions of *Tetrahymena* TERT have affinity for G-quadruplex DNA. (A) Schematic representation of *T. thermophila* TERT with its conserved telomerase- and RT-specific domains. Motif 1, marked in red, marks the location of the K538A mutation. Solid bars outline the fragments of the protein that were tested in DNA binding assays. (B) The dissociation constants of TERT and its fragments in the presence or absence of RNA for the 12GT tetramer (T) or denatured (D) DNA primer. The values are presented as the means ± standard deviations of 2−4 experiments. * indicates that the K_d is above the maximal concentration of tetramer (20 μM) tested. "bg" indicates that no specific interaction above the background level was detected at up to 5 μM primer concentration. ND, not determined.

that of the nontelomeric primer of random sequence (Bio-PBR). The affinity of telomerase for G-quadruplex DNA as measured by this direct binding assay (K_d) was within 3-fold of that measured by activity assays (K_m).

TER Is Not Required for G-Quadruplex Interaction with TERT. Having established that the in vitro reconstituted TERT and TER telomerase complex readily binds to and extends the parallel four-stranded G-quadruplex, we sought to determine whether TER is required for this interaction. DNA binding assays were carried out with telomerase that was in vitro reconstituted in the absence of the RNA component. A pull-down of TERT with biotinylated 12GT tetramer in the absence of TER revealed that the RNA component is not necessary for the interaction (Supporting Information, Figure 2). The same result was obtained for the denatured counterpart. In agreement, both human and *Tetrahymena* recombinant TERT have been demonstrated to bind to linear DNA in an RNA-independent manner (*33*; S. Finger and T. Bryan, submitted for publication). This is consistent with the existence of one or more protein-based "anchor" site(s). The dissociation constant (K_d) of TERT for 12GT tetramer in the absence of RNA is much higher than that in its presence (Figure 4B, top row), suggesting that the RNA component improves the strength of the interaction. In contrast, for the denatured form of the quadruplex, the K_d values in the presence or absence of RNA are not as disparate (Figure 4B, second row). It is conceivable that the RNA may induce a conformational change in TERT, which would allow the protein to better accommodate G-quadruplex binding.

Both N- and C-Terminal Regions of TERT Bind the Four-Stranded Parallel G-Quadruplex. To localize regions of the TERT protein involved in G-quadruplex interaction, the roles of the telomerase- and reverse-transcriptase specific TERT domains in binding to the four-stranded G-quadruplex were investigated using the direct DNA binding assay (Figure 4). The N-terminal TERT fragment comprising amino acids 1−519 (N519) had near full-length affinity for quadruplex DNA in both the presence or the absence of TER; the same was true for the C-terminal fragment that constitutes amino acids 520−1117 of TERT (C598) (Figure 4B). The binding was specific for telomeric DNA (i.e., above the level of binding for nontelomeric PBR primer). Like the full length protein, both the N- and the C-terminal fragments also bound to the denatured form of the quadruplex. However, the absence of the RNA component did not result in reduced affinity, as it did in the case of full length TERT. It has been shown that TERT interaction with the RNA moiety is primarily mediated through the N-terminal N519 fragment, with C598 contributing negligibly to this interaction (*28*, *34*, *35*; S. Finger and T. Bryan, submitted for publication). Therefore, it is plausible that any putative conformational change that the RNA imparts onto TERT is mediated through N519 but lies in the C-terminal half of the protein. In such a scenario, the absence of RNA will not affect the binding affinity of either half of TERT to DNA, which is what we observe.

A region of the protein at the extreme N terminus of TERT encompassing amino acids 2−191, designated as the TEN domain, is essential for telomerase activity and contains amino acids that are involved in interactions with linear DNA (*29*, *36*). This region alone appears to have minimal affinity for the quadruplex; we could not detect interaction of the TEN domain with DNA above background level. This pattern of binding closely mimics that of a linear DNA primer in the absence of salt (S. Finger and T. Bryan, submitted for publication), indicating that TEN exhibits a binding affinity that is beyond the limit of detection of our assay. Thus, while this domain has been shown to cross-link to DNA (*36*; S. Finger and T. Bryan, submitted for publication), in isolation, it does not provide much of the binding affinity. Collectively, the results show that both major domains of TERT specifically and independently bind the four-stranded parallel G-quadruplex independently of TER.

The Four-Stranded Parallel G-Quadruplex Reduces the Affinity of Telomerase for dTTP. To determine whether the G-quadruplex affects the nucleotide binding capability of wild-type telomerase, the efficiency of nucleotide incorporation was tested in the presence of G-quadruplex, denatured, or linear DNA. Because the 12GT oligonucleotide that forms the four-stranded G-quadruplex terminates with "GT" at its 3′-end, the first nucleotide to be added during its extension is a thymidine residue. To capture the moment of initial nucleotide addition, we performed an "initiation reaction", whereby the DNA substrate (12GT tetramer, denatured or linear) was incubated in the presence of telomerase, ddGTP, and α^{32}P-dTTP (Figure 5A). The predicted pattern of extension is that a "T" residue followed by a terminal "G" residue are added, resulting in products of the length of the primer plus one or two nucleotides. Curiously, wild-type telomerase failed to incorporate dTTP in the presence of the four-stranded parallel G-quadruplex but, as expected, was

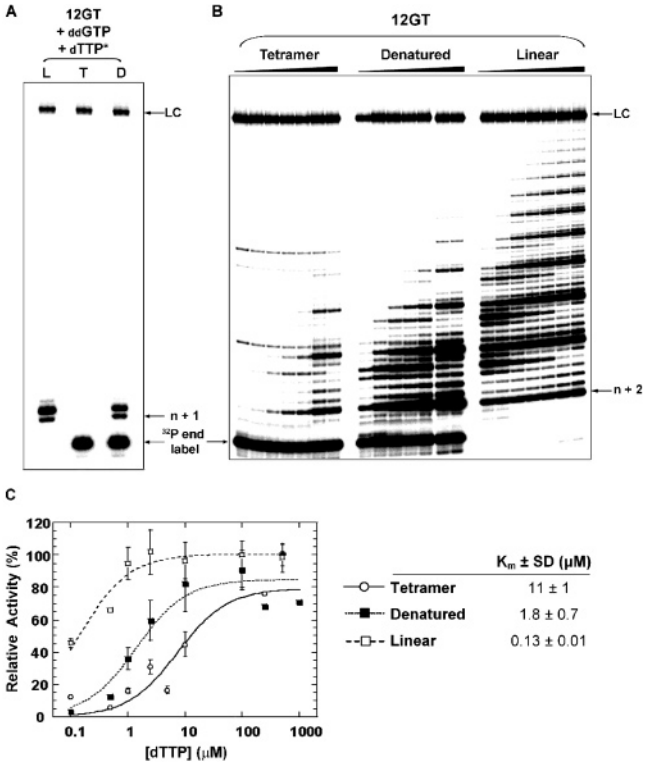


FIGURE 5: Telomerase has reduced affinity for dTTP in the presence of 12GT four-stranded G-quadruplex. (A) Initiation reaction: ^{32}P end-labeled gel-purified 12GT tetramer (T) and denatured (D) and linear (L) controls at 12 μM concentration were incubated in the presence of ddGTP, [α ^{32}P]dTTP, and recombinant telomerase to assess the incorporation of the first two nucleotides. "n+1" signifies the first "T" residue incorporated into the primer. (B) ^{32}P end-labeled gel-purified 12GT tetramer and denatured and linear controls at 10 μM concentration were incubated in the presence of recombinant telomerase, 10 μM dGTP (9.7 μM dGTP + 0.3 μM [α^{32}P]dGTP), and increasing concentrations of dTTP (0.5, 1, 2.5, 5, 10, 100, and 500 μM) to determine the affinity of telomerase for dTTP in the presence of G-quadruplex vs unstructured substrate. "n+2" indicates the first two nucleotides incorporated into the primer, and "LC" is a ^{32}P-labeled 100-mer DNA oligonucleotide used as a recovery and loading control. (C) Telomerase activity was quantitated from a gel such as the one shown in B and plotted as a percentage of maximal value against dTTP concentration. From this plot, the affinity constant K_m of telomerase for dTTP was derived. The K_m values are means $\pm$ standard deviations from three to four independent experiments.

able to do so in the presence of denatured or linear DNA. Because the concentration of dTTP (1.3 μM) is much lower in this assay than in the standard extension assay (100 μM; due to the use of α^{32}P-dTTP), this is suggestive of a nucleotide incorporation defect imposed by the quadruplex. To test this idea, the affinity of wild-type telomerase for dTTP in the presence of saturating concentrations (10−20 μM) of G-quadruplex, denatured, or linear DNA was measured by carrying out telomerase activity assays at a range of dTTP concentrations (Figure 5B). The K_m of telomerase for dTTP was dramatically increased in the presence of 12GT tetramer as compared to its denatured and linear controls (Figure 5C). There is a severe reduction in the dTTP binding affinity of telomerase in the presence of denatured as compared with linear DNA (Figure 5C); because the only difference between these samples is the presence or absence of NaCl, we conclude that the salt affects dTTP binding affinity. The results suggest that a bulky four-stranded G-quadruplex imposes a conformational change on the nucleotide binding pocket of telomerase.

A Point Mutation in Motif 1 of the TERT RT Domain Reduces Telomerase Extension of Four-Stranded G-Quadruplex. To further refine regions of TERT that may be specifically involved in G-quadruplex extension, a panel of 13 previously characterized point mutants of *Tetrahymena* TERT (*28*, *37*) was screened for the ability to extend the four-stranded parallel G-quadruplex. The ability to extend unstructured forms of the DNA (linear and denatured) but not the quadruplex form would reveal a region of TERT involved in binding, resolution, or extension of the G-quadruplex structure. Indeed, a mutant of telomerase bearing a single lysine to alanine point mutation in motif 1 of *Tetrahymena* TERT, at amino acid 538 (K538A), was found to possess these properties (Figure 6). The K538A mutant extends linear 12GT and the denatured form of the quadruplex, albeit less efficiently than wild-type enzyme. However, the ability of K538A to extend the 12GT tetramer is considerably reduced and constitutes only 5 $\pm$ 3% of denatured control, while extension of 12GT tetramer by wild-type telomerase is 50 $\pm$ 4% of denatured control. As an additional control, another telomerase point mutant immediately adjacent to K538A (K539A) was tested; it does not exhibit such dramatic loss of ability to extend the G-quadruplex substrate (Figure 6A). The remaining 11 mutants, L327A, K329A, C331A, P334A, R543A, I545A, Y623A, D624A, S778A, K849A, and S880A, also did not exhibit any phenotype specific to elongation of G-quadruplex DNA (data not shown). These results identify a localized region of TERT RT motif 1 as being specifically involved in G-quadruplex extension.

To test whether the loss of quadruplex extension could be attributed to an effect on binding to quadruplex DNA, binding assays with wild-type and K538A mutant forms of the enzyme were performed. K538A mutant enzyme bound to G-quadruplex DNA with a 2-fold weaker affinity than that of wild-type enzyme (Figure 6B,C). This is not sufficient to fully account for the 10-fold reduction in the ability of the mutant to extend the quadruplex. The affinities of both wild-type and mutant forms of the enzyme for the denatured form of the quadruplex were equivalent (Figure 6B,C).

The K538A mutation in *Tetrahymena* TERT is known to cause reduced nucleotide binding affinity in telomerase, so that the efficiency of dGTP and dTTP incorporation is compromised (*37*). This defect in combination with intrinsically reduced affinity of telomerase for dTTP in the presence of the four-stranded G-quadruplex may explain the inability of K538A mutant to extend the quadruplex. This implicates the active site of TERT as a region directly involved in recognition of a G-quadruplex substrate.

DISCUSSION

Here, we present evidence that the mechanism with which ciliate telomerase recognizes and extends G-quadruplex DNA is distinct from that used for a linear DNA substrate and that this property of telomerase is afforded by a localized region within its protein component, TERT.

We have characterized a novel parallel four-stranded G-quadruplex conformation that arises from the 12GT *Tetrahymena* telomeric sequence and which efficiently

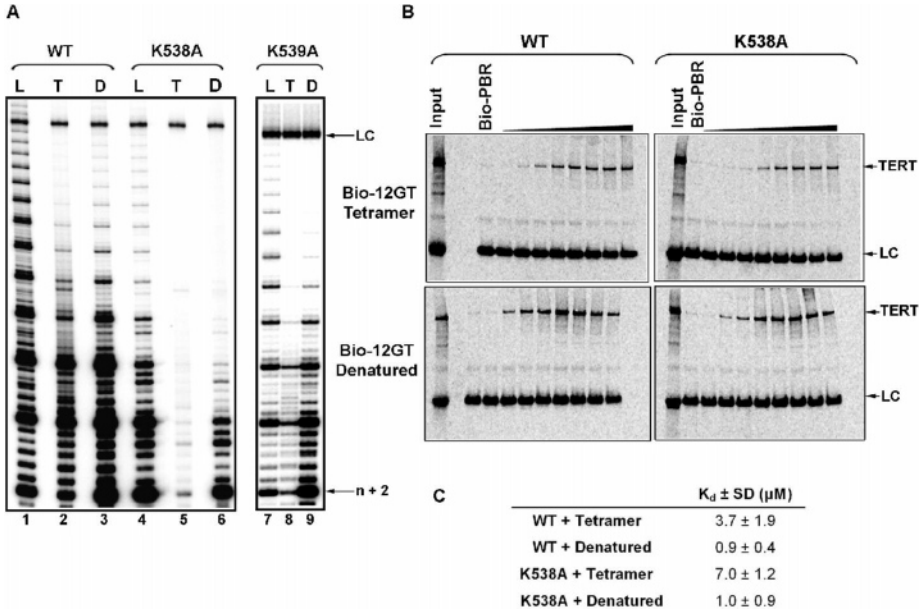


FIGURE 6: K538A mutant telomerase loses the ability to extend 12GT four-stranded G-quadruplex. (A) Direct primer extension assay using ^{32}P end-labeled gel-purified 12GT tetramer (T; lanes 2, 5, and 8) or its denatured (D; lanes 3, 6, and 9) and linear (L; lanes 1, 4, and 7) counterparts and recombinant wild-type (wt; lanes 1−3), K538A (lanes 4−6), or K539A (lanes 7−9) mutants of telomerase. The DNA concentration in lanes 1−6 and 7−9 is 15 and 8 μM, respectively. "n+2" indicates the first two nucleotides added to the primer, and "LC" is a ^{32}P-labeled 100-mer DNA oligonucleotide used as a recovery and loading control. (B) DNA binding assay to directly measure the affinity of wild-type vs mutant K538A telomerase for G-quadruplex. Biotinylated gel-purified 12GT tetrameric G-quadruplex and its denatured counterpart at 0, 0.2, 0.5, 1, 2.5, 10, 15, and 20 μM concentrations or nontelomeric control (Bio-PBR) at 2.5 μM were incubated with ^{35}S-methionine labeled full-length wt or mutant recombinant telomerase (in the presence of RNA) and recovered on NeutrAvidin beads. "Input" represents 50% of starting material in the bound lanes. "LC" is a ^{33}P-labeled PBR48-Bio oligonucleotide used as a loading and recovery control. (C) The dissociation constant K_d of wild-type and mutant telomerase for 12GT tetramer and denatured counterpart is expressed as a mean ± standard deviation of three independent experiments.

primes both recombinant and native telomerase in vitro. NMR analysis had previously revealed the solution structure for the G-quadruplex that assembles from this sequence to be an antiparallel dimer that interconverts between "head-to-head" and "head-to-tail" configurations in a Na^+-containing solution (*32*). We had previously demonstrated by native gel electrophoresis, circular dichroism, and UV cross-linking analyses that an additional higher molecular weight parallel structure forms (*23*). Electrospray ionization mass spectrometry in the present work reveals this to be a tetramolecular G-quadruplex. Although the precise architecture of telomeric DNA in *T. thermophila* in vivo is largely unknown and a parallel four-stranded G-quadruplex has not been directly observed at telomeres of ciliated protozoa, the potential exists for its formation in cells. First, the majority of 3′ overhangs in *Tetrahymena* are 14−15 or 20−21 bases in length (*38*), suggesting that formation of an intermolecular structure would be favored, since intramolecular G-quadruplex formation requires at least four telomeric repeats. Second, during meiotic prophase I, the chromosomes in *T. thermophila* have been shown to be arranged in parallel bundles within the micronuclear crescent (a threadlike structure that forms in the early stages of micronuclear differentiation), resulting in telomere clustering at one end of the crescent (*39*). Therefore, it is conceivable that assembly of a parallel four-stranded G-quadruplex at telomeres could facilitate telomere clustering during the early stages of micronuclear differentiation. The existence of such a stable G-quadruplex, however, may require a resolvase to promote subsequent separation of the chromosomes.

The four-stranded G-quadruplex is highly stable (with an undetectable dissociation rate at 25 °C and a half-life in the order of days at 37 °C); yet, it is readily extended by both immunopurified recombinant and native *Tetrahymena* telomerase (*23* and present work). We have also previously demonstrated that the 12GT G-quadruplex is refractory to extension by terminal transferase (*23*), a polymerase known to catalyze the elongation of single- or double-stranded DNA. This, together with the robust stability of the quadruplex, discounts the argument that quadruplex extension by telomerase (which was carried out for only 15 min at 25 °C) can be attributed to spontaneous unfolding of this structure. Although telomerase has greater affinity for a denatured or linear substrate, the affinity for G-quadruplex DNA is considerable.

To explore the biochemical basis underlying extension of this stable higher order structure by telomerase, we sought to determine whether there was a set of interaction sites within TERT that were unique to G-quadruplex assembled DNA, that is, whether there were regions within the protein that specifically promoted extension of the parallel four-stranded G-quadruplex but not its unstructured counterpart. We found two neighboring single amino acid substitutions, K538A and K539A, in RT motif 1 of TERT (see Figure 4A), both of which could support the catalytic extension of linear DNA, but only one of which, K539A, was able to support extension of the quadruplex primer. K538A mutant telomerase had severely compromised ability to extend 12GT tetramer while retaining almost wild-type binding affinity for this structure.

K538 is an amino acid that is conserved in almost all TERTs and other RTs (*18*, *40*). In the human immunodeficiency virus-1 (HIV-1) RT crystal structure (*41*, *42*), the corresponding lysine residue (K65) is located in a protruding

loop between two β-sheets that form the tip of the "fingers" domain at the edge of the enzyme active site (*41*). The template-primer duplex sits in the pocket between the "fingers" and "palm" domains, so it is conceivable that this amino acid is involved in positioning the G-quadruplex in the active site. A single-stranded DNA substrate, which is less bulky, may not need to form the same contacts at the edge of the active site and, hence, would be less sensitive to a substitution at this position.

When an incoming nucleotide is positioned in the active site, HIV-1 RT undergoes a conformational change such that the "fingers" close in on the "palm", and K65 now donates hydrogen bonds to the γ-phosphate of the incoming nucleotide (*42*). This may explain why K538A in *Tetrahymena* TERT is defective in nucleotide usage (*37*). The K65A mutation in HIV-1 RT only moderately affected polymerase activity but was shown to significantly reduce the conformational flexibility of the dNTP binding pocket, making it more rigid (*43*). Thus, it is plausible that the K538A mutation results in a similar outcome, whereby the now "rigid" nucleotide binding pocket does not have the flexibility to accommodate a structured substrate. It is also possible that at this stage of the catalytic cycle, decreased nucleotide affinity in the mutant combines with the inherently lower nucleotide affinity when a G-quadruplex is a substrate, resulting in a specific defect in elongating G-quadruplexes.

The exceedingly stable nature of the 12GT four-stranded G-quadruplex, its ability to impart a conformational change on the nucleotide binding pocket of telomerase, and the selective impairment of its extension by a point mutant of the enzyme strongly suggest that G-quadruplex DNA is a substrate for telomerase while it is structured. At the same time, correct alignment of G-quadruplex substrate with the RNA template is observed (Figure 5A; data not shown). On the basis of these observations, we propose that telomerase-driven partial resolution of 12GT G-quadruplex must precede its extension by this enzyme.

In summary, we find that a single amino acid substitution within TERT specifically alters the ability of telomerase to process G-quadruplex DNA and that G-quadruplex extension incurs lowered nucleotide affinity. These findings are inconsistent with a model in which the G-quadruplex is spontaneously dissociating prior to extension by telomerase, further validating our observation that intermolecular G-quadruplex DNA is a viable substrate for wild-type telomerase. Our observations also suggest that utilization of a four-stranded G-quadruplex as a substrate results in changes in the conformation of the enzyme's active site. Thus, telomerase recognizes G-quadruplex DNA as being distinct from a linear substrate, and this conformation-dependent recognition may be mediated by the active site of the enzyme. It will be interesting to determine whether the ability of telomerase to recognize and extend structured DNA is conserved across species and to assess the relevance of these in vitro observations to in vivo processes.

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SUPPORTING INFORMATION AVAILABLE

Demonstration that telomerase does not hydrolyze ribo- or deoxyribonucleotides in the presence of G-quadruplex substrate or its denatured form and the associated experimental protocol for this figure. Depiction that TER is not necessary for TERT interaction with its substrate. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Blackburn, E. H. (1984) The molecular structure of centromeres and telomeres, *Annu. Rev. Biochem. 53*, 163−194.
2. Blackburn, E. H., and Gall, J. G. (1978) A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in *Tetrahymena*, *J. Mol. Biol. 120*, 33−53.
3. Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H., and de Lange, T. (1999) Mammalian telomeres end in a large duplex loop, *Cell 97*, 503−514.
4. de Lange, T. (2004) Opinion: T-loops and the origin of telomeres, *Nat. Rev. Mol. Cell Biol. 5*, 323−329.
5. Schaffitzel, C., Berger, I., Postberg, J., Hanes, J., Lipps, H. J., and Pluckthun, A. (2001) *In vitro* generated antibodies specific for telomeric guanine-quadruplex DNA react with *Stylonychia lemnae macronuclei*, *Proc. Natl. Acad. Sci. U.S.A. 98*, 8572−8577.
6. Paeschke, K., Simonsson, T., Postberg, J., Rhodes, D., and Lipps, H. J. (2005) Telomere end-binding proteins control the formation of G-quadruplex DNA structures in vivo, *Nat. Struct. Mol. Biol. 12*, 847−854.
7. Williamson, J. R., Raghuraman, M. K., and Cech, T. R. (1989) Monovalent cation-induced structure of telomeric DNA: The G-quartet model, *Cell 59*, 871−880.
8. Williamson, J. R. (1994) G-quartet structures in telomeric DNA, *Annu. Rev. Biophys. Biomol. Struct. 23*, 703−730.
9. Zhang, N., Phan, A. T., and Patel, D. J. (2005) (3 + 1) Assembly of three human telomeric repeats into an asymmetric dimeric G-quadruplex, *J. Am. Chem. Soc. 127*, 17277−17285.
10. Xu, Y., Noguchi, Y., and Sugiyama, H. (2006) The new models of the human telomere d[AGGG(TTAGGG)$_3$] in K^+ solution, *Bioorg. Med. Chem. 14*, 5584−5591.
11. Ambrus, A., Chen, D., Dai, J., Bialis, T., Jones, R. A., and Yang, D. (2006) Human telomeric sequence forms a hybrid-type intramolecular G-quadruplex structure with mixed parallel/antiparallel strands in potassium solution, *Nucleic Acids Res. 34*, 2723−2735.
12. Luu, K. N., Phan, A. T., Kuryavyi, V., Lacroix, L., and Patel, D. J. (2006) Structure of the human telomere in K^+ solution: An intramolecular (3 + 1) G-quadruplex scaffold, *J. Am. Chem. Soc. 128*, 9963−9970.
13. Greider, C. W., and Blackburn, E. H. (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts, *Cell 43*, 405−413.
14. Shippen-Lentz, D., and Blackburn, E. H. (1990) Functional evidence for an RNA template in telomerase, *Science 247*, 546−552.
15. Weinrich, S. L., Pruzan, R., Ma, L., Ouellette, M., Tesmer, V. M., Holt, S. E., Bodnar, A. G., Lichtsteiner, S., Kim, N. W., Trager, J. B., Taylor, R. D., Carlos, R., Andrews, W. H., Wright, W. E., Shay, J. W., Harley, C. B., and Morin, G. B. (1997) Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT, *Nat. Genet. 17*, 498−502.
16. Collins, K., and Gandhi, L. (1998) The reverse transcriptase component of the *Tetrahymena* telomerase ribonucleoprotein complex, *Proc. Natl. Acad. Sci. U.S.A. 95*, 8485−8490.
17. Beattie, T. L., Zhou, W., Robinson, M. O., and Harrington, L. (1998) Reconstitution of human telomerase activity *in vitro*, *Curr. Biol. 8*, 177−180.
18. Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Lingner, J., Harley, C. B., and Cech, T. R. (1997) Telomerase catalytic subunit homologs from fission yeast and human, *Science 277*, 955−959.
19. Lingner, J., Hughes, T. R., Shevchenko, A., Mann, M., Lundblad, V., and Cech, T. R. (1997) Reverse transcriptase motifs in the catalytic subunit of telomerase, *Science 276*, 561−567.

20. Shay, J. W., and Bacchetti, S. (1997) A survey of telomerase activity in human cancer, *Eur. J. Cancer 33*, 787−791.
21. Zahler, A. M., Williamson, J. R., Cech, T. R., and Prescott, D. M. (1991) Inhibition of telomerase by G-quartet DNA structures, *Nature 350*, 718−720.
22. Oganesian, L., and Bryan, T. M. (2007) Physiological relevance of telomeric G-quadruplex formation: A potential drug target, *BioEssays 29*, 155−165.
23. Oganesian, L., Moon, I. K., Bryan, T. M., and Jarstfer, M. B. (2006) Extension of G-quadruplex DNA by ciliate telomerase, *EMBO J. 25*, 1148−1159.
24. Larsen, M. R., Graham, M. E., Robinson, P. J., and Roepstorff, P. (2004) Improved detection of hydrophilic phosphopeptides using graphite powder microcolumns and mass spectrometry: Evidence for *in vivo* doubly phosphorylated dynamin I and dynamin III, *Mol. Cell. Proteomics 3*, 456−465.
25. Raghuraman, M. K., and Cech, T. R. (1990) Effect of monovalent cation-induced telomeric DNA structure on the binding of *Oxytricha* telomeric protein, *Nucleic Acids Res. 18*, 4543−4552.
26. Bryan, T. M., Goodrich, K. J., and Cech, T. R. (2000) A mutant of *Tetrahymena* telomerase reverse transcriptase with increased processivity, *J. Biol. Chem. 275*, 24199−24207.
27. Bryan, T. M., Goodrich, K. J., and Cech, T. R. (2003) *Tetrahymena* telomerase is active as a monomer, *Mol. Biol. Cell 14*, 4794−4804.
28. Bryan, T. M., Goodrich, K. J., and Cech, T. R. (2000) Telomerase RNA bound by protein motifs specific to telomerase reverse transcriptase, *Mol. Cell 6*, 493−499.
29. Jacobs, S. A., Podell, E. R., Wuttke, D. S., and Cech, T. R. (2005) Soluble domains of telomerase reverse transcriptase identified by high-throughput screening, *Protein Sci. 14*, 2051−2058.
30. Greider, C. W., and Blackburn, E. H. (1987) The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity, *Cell 51*, 887−898.
31. Lingner, J., and Cech, T. R. (1996) Purification of telomerase from *Euplotes aediculatus*: Requirement of a primer 3′ overhang, *Proc. Natl. Acad. Sci. U.S.A. 93*, 10712−10717.
32. Phan, A. T., Modi, Y. S., and Patel, D. J. (2004) Two-repeat *Tetrahymena* telomeric d(TGGGGTTGGGGT) sequence interconverts between asymmetric dimeric G-quadruplexes in solution, *J. Mol. Biol. 338*, 93−102.
33. Wyatt, H. D., Lobb, D. A., and Beattie, T. L. (2007) Characterization of physical and functional anchor site interactions in human telomerase, *Mol. Cell. Biol. 27*, 3226−3240.
34. Lai, C. K., Mitchell, J. R., and Collins, K. (2001) RNA binding domain of telomerase reverse transcriptase, *Mol. Cell. Biol. 21*, 990−1000.
35. O'Connor, C. M., Lai, C. K., and Collins, K. (2005) Two purified domains of telomerase reverse transcriptase reconstitute sequence-specific interactions with RNA, *J. Biol. Chem. 280*, 17533−17539.
36. Jacobs, S. A., Podell, E. R., and Cech, T. R. (2006) Crystal structure of the essential N-terminal domain of telomerase reverse transcriptase, *Nat. Struct. Mol. Biol. 13*, 218−225.
37. Miller, M. C., Liu, J. K., and Collins, K. (2000) Template definition by *Tetrahymena* telomerase reverse transcriptase, *EMBO J. 19*, 4412−4422.
38. Jacob, N. K., Skopp, R., and Price, C. M. (2001) G-overhang dynamics at *Tetrahymena* telomeres, *EMBO J. 20*, 4299−4308.
39. Loidl, J., and Scherthan, H. (2004) Organization and pairing of meiotic chromosomes in the ciliate *Tetrahymena thermophila*, *J. Cell Sci. 117*, 5791−5801.
40. Friedman, K. L., Heit, J. J., Long, D. M., and Cech, T. R. (2003) N-terminal domain of yeast telomerase reverse transcriptase: Recruitment of Est3p to the telomerase complex, *Mol. Biol. Cell 14*, 1−13.
41. Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H., and Arnold, E. (1993) Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA, *Proc. Natl. Acad. Sci. U.S.A. 90*, 6320−6324.
42. Huang, H., Chopra, R., Verdine, G. L., and Harrison, S. C. (1998) Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: Implications for drug resistance, *Science 282*, 1669−1675.
43. Harris, D., Kaushik, N., Pandey, P. K., Yadav, P. N., and Pandey, V. N. (1998) Functional analysis of amino acid residues constituting the dNTP binding pocket of HIV-1 reverse transcriptase, *J. Biol. Chem. 273*, 33624−33634.

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