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Structure of Long Human Telomeric RNA (TERRA): G-Quadruplexes Formed by Four and Eight UUAGGG Repeats Are Stable Building Blocks

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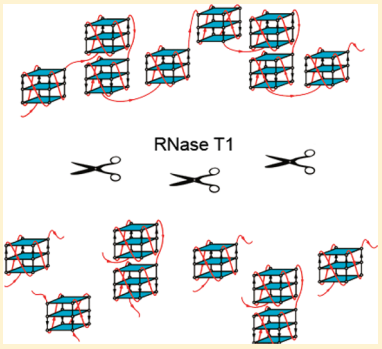
Supporting Information

ABSTRACT: The discovery of long RNA transcripts of telomeric repeats (TERRA) and their potential to form G-quadruplexes stimulated studies on the possible arrangements of G-quadruplexes along TERRA. Here we performed ribonuclease protection assay to investigate the structures formed by long human TERRA. We found that G-quadruplexes comprising four and eight UUAGGG repeats were most resistant to RNase T1 digestion, presumably with the former adopting an all-parallel-stranded propeller-type conformation and the latter forming a structure with two tandemly stacked G-quadruplex subunits each containing three G-tetrad layers. Molecular dynamics simulations of eight-repeat human TERRA sequences consisting of different stacking interfaces between the two G-quadruplex subunits, i.e., 5′−5′, 3′−3′, 3′−5′, and 5′−3′, demonstrated stacking feasibility for all but the 5′−3′ arrangement. A continuous stacking of the loop bases from one G-quadruplex subunit to the next was observed for the 5′−5′ stacking conformation. We also put forward other possible stacking arrangements that involve more than one linker connecting the two G-quadruplex subunits. On the basis of these results, we propose a "beads-on-a-string"-like arrangement along human TERRA, whereby each bead is made up of either four or eight UUAGGG repeats in a one- or two-block G-quadruplex arrangement, respectively.



Guanine-rich nucleic acids can form a four-stranded structure called the G-quadruplex, based on the stacking of multiple G·G·G·G tetrads.[1−3] Biological roles of G-quadruplex structures have been discussed for G-rich DNA in various genomic locations,[4] such as telomeres,[5] oncogenic promoters,[6,7] and immunoglobulin switches,[4] as well as for G-rich RNA at 5′-untranslated regions of several oncogenes.[8]

Telomeres, which are located at the chromosomal ends, act as protective caps that prevent chromosome loss and degradation. Telomeres had always been thought to be transcriptionally silent until the recent finding that they could be transcribed into RNA molecules with lengths ranging from 100 to 9000 nt.[9,10] It has further been shown that telomeric-repeat-containing RNA (TERRA) perform various cellular regulatory functions, such as regulation of telomere length,[10−12] inhibition of telomerase,[13] telomeric heterochromatin formation,[14,15] and telomere protection.[9,16−18] Similar to the DNA counterparts, short G-rich human telomeric RNA sequences were shown to form G-quadruplex structures.[19−29] It has been suggested that the structures of long human telomeric DNA and RNA sequences are based on multiple G-quadruplex blocks, each formed by a four-repeat segment.[20−22,29−34] So far, high-resolution structures could only be obtained for RNA sequences containing up to two human telomeric repeats.[21,25] Here, we address the question on the arrangement of G-quadruplexes in long human TERRA sequences (of up to 96 repeats) by performing ribonuclease protection assays and molecular dynamics (MD) simulations. We found that G-quadruplexes formed by four and eight human telomeric UUAGGG repeats are abundant and stable structural units that may serve as building blocks in long human TERRA, and loop−loop interactions may contribute to the stability of higher-order assemblies.

MATERIALS AND METHODS

Construction of Plasmids and DNA Templates. The PRST5 plasmid[35] containing 96 human telomeric repeats (Figure S1, Supporting Information) was a gracious gift from Jack D. Griffith's laboratory.

Another plasmid containing 9 human telomeric repeats (Figure S1, Supporting Information) was constructed according to previously published protocol[35] with some modifications. A DNA template containing nine human telomeric repeats 5′-CCCGGATCCGTCTCA(CCCTAA)$_9$GTCTTCAAGCTT-CCC-3′ was used for PCR reaction. Oligonucletide containing BbsI and HindIII restriction sites 5′-GGGAAGCTTGAAGACTT-3′ was used as the forward primer. Another oligonucleotide containing BsmBI and BamHI restrictions sites 5′-CCCGGATC-CGTCTCACC-3′ was used as the reverse primer. All the oligonucleotides were synthesized in house on an ABI 394 DNA

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synthesizer. The PCR product was cloned into pBluescript II SK+ (1st Base, Singapore) using HindIII and BamHI restriction sites (Fermentas) according to the standard protocol. The reaction mixture was then transformed into *E. coli* DH5α cells. Blue/white screening was performed to select the colonies transformed with the cloned telomeric repeats and confirmed by sequencing (1st Base, Singapore).

DNA templates coding for 12 repeats of the wild-type and mutated human telomeric sequences (Figure S1, Supporting Information) were synthesized using an ABI DNA synthesizer.

***In Vitro* Transcription.** Plasmids containing either 9 or 96 human telomeric repeats were linearized using BamHI or BbsI and purified using either agarose gel extraction kit or PCR purification kit (Qiagen Inc.). *In vitro* transcription was performed on linearized plasmids or synthetic DNA templates using T7 RNA Polymerase (Fermentas). The transcription product was compared with known reference marker on gel to estimate the transcription yield. For 12-repeat sequences, the DNA templates were removed by RQ1-DNase (Promega) digestion followed by acidic phenol−chloroform extraction and ethanol precipitation.

RNA Structure Formation. The transcription product, with or without DNA template removal, was diluted to about 200 ng/μL in 100 mM KCl and 50 mM potassium phosphate (pH 7.0). The samples were then either annealed (by heating to 100 °C for 2 min and slowly cooling down to room temperature) or simply incubated at room temperature overnight.

Digestion Assay and Gel Electrophoresis. In ribonuclease protection assay, the RNA transcripts were incubated with different amounts of RNase T1 (Fermentas) for 1 h at room temperature. The amount of RNA transcripts used in each reaction tube was 600 ng. The amount of RNase T1 varied as indicated in the captions of Figure 1 and Figures S2−S4 of the Supporting Information. To denature the RNA products, an equal volume of denaturing gel loading solution (8 M urea, 40% sucrose) was added to the reaction mixture, and the samples were then heated for 10 min at 100 °C. Samples were analyzed by denaturing gel electrophoresis performed on a 86 mm × 68 mm × 0.75 mm 8 M urea/6% polyacrylamide gel in 1 × TBE buffer (pH 8.3). 12-nt r[UAGGGUUAGGGU], 23-nt r[UAGGG-(UUAGGG)$_3$], and 51-nt r[(UUAGGG)$_8$UUA] synthetic human telomeric RNA sequences, purchased from Dharmacon, were loaded as reference markers. The gel was stained with SYBR Gold (Invitrogen) and revealed using UV transilluminator (Alpha Innotech).

Nuclear Magnetic Resonance. NMR spectra of the synthetic 8-repeat human telomeric RNA sequence r[(UUAGGG)$_8$UUA] were recorded on a 600 MHz Bruker spectrometer equipped with a CryoProbe using JR-type pulse sequences for water signal suppression.[36,37] RNA strand concentration was 50 μM. The sample contained 10 mM KCl and 10 mM potassium phosphate (pH 7.0).

Circular Dichroism. CD spectra of the synthetic 8-repeat human telomeric RNA sequence r[(UUAGGG)$_8$UUA] were recorded on a Jasco J-815 spectropolarimeter using a 1 cm path length quartz cuvette in a reaction volume of 500 μL. RNA strand concentration was 2 μM. The sample contained 10 mM KCl and 10 mM potassium phosphate (pH 7.0). Scans from 220 to 320 nm were performed with 200 nm/min scanning speed. For each spectrum, an average of two scans was taken, spectral contribution from the buffer was subtracted.

Melting Experiments. Thermal stability of 4-, 8-, and 9-repeat TERRA sequences in K^+ solution, containing 20 mM potassium phosphate (pH 7) and 50 mM KCl, was characterized in UV melting experiments. The absorbance at 295 nm was measured as a function of temperature on a Jasco V-650 UV−vis spectrophotometer using quartz cuvettes with 1 cm path length. The data were zero-corrected using the baseline absorption measurement at 330 nm. RNA concentrations ranged from 1 to 4 μM. The temperature ranged from 20 to 95 °C; cooling and heating rates were 0.2 °C/min. The melting point (T_m) was defined as the temperature where 50% of the sample was unfolded.

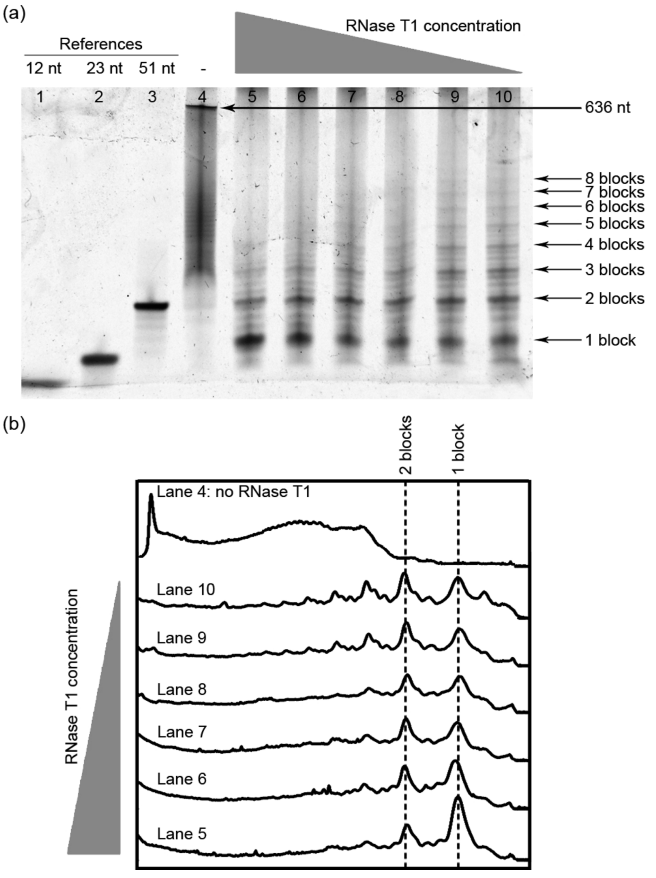

Figure 1. Long human TERRA digested with varying concentrations of RNase T1. (a) Denaturing PAGE of TERRA upon digestion with different concentrations of RNase T1 (lane 5: 200 u; lane 6: 100 u; lane 7: 50 u; lane 8: 30 u; lane 9: 6 u; and lane 10: 1 u) at 25 °C for 1 h. Lane 4 contains undigested TERRA. Lanes 1−3 contain 12-nt, 23-nt, and 51-nt synthetic TERRA markers, respectively. The previous assignment of the one-block band to a 28-nt RNA fragment[22] is consistent with its slightly slower migration as compared to the 23-nt synthetic TERRA marker. (b) Histogram showing the intensity across the length of the gel for the lanes containing undigested (lane 4) and digested (lanes 5−10) TERRA. The 636-nt RNA transcript contains 96 UUAGGG repeats (576 nt) and flanking sequences at both 5′ and 3′ ends (Figure S1, Supporting Information). Partially degraded or incompletely transcribed RNA were observed together with the full-length RNA (lane 4).

Molecular Dynamics Simulations. Molecular dynamics (MD) simulations were performed using the program AMBER 10[38] with the force field ParmBSC0.[39] Initial structures of one block of the parallel-stranded propeller-type G-quadruplex were taken from a crystal structure (PDB ID: 3IBK) or a NMR structure (PDB ID: 2KBP); the dimers were built using PyMOL[40] and the xleap module of AMBER. K^+ ions have been added between G-tetrads. The generated dimers were first energy-minimized in

vacuo with 1000 steps (500 steps of steepest descent followed by 500 steps of conjugate gradient) to remove any steric clashes. The system was neutralized by K^+ ions (totally 44 K^+ ions) and solvated with 8715, 9289, 10 989, and 10 670 TIP3P[41] water molecules for the 5′−5′, 3′−5′, 5′−3′ and 3′−3′ stacking simulations, respectively, in a truncated octahedral box. Simulations were performed with periodic boundary conditions at constant temperature (300 K) and pressure (1 bar) using the Berendsen algorithm.[42] The integration time step was 2 fs, and covalent bonds involving hydrogens were restrained using SHAKE.[43] Long-range electrostatic interactions were treated using the particle mesh Ewald (PME) approach[44] with a 9 Å direct space cutoff. The Lennard-Jones interactions were cut off at a distance of 9 Å. The nonbonded pair list was updated heuristically and the center-of-mass motion was removed every 10 ps.

Water molecules and counterions were energy-minimized and equilibrated at 100 K around the fixed DNA for 100 ps in the NVT (at constant volume and temperature) ensemble; the entire system was then heated from 100 to 300 K in 10 ps by 5 K increments with harmonic positional restraints on the solute atoms (force constant of 5.0 kcal/(mol Å^2)). The simulation was continued in NPT (at constant pressure and temperature). The positional restraints were gradually removed over 250 ps. Structures were taken from the MD simulations every picosecond for further analyses.

Four simulations were performed: a dimer with a 5′−5′ stacking interface, 3′−5′ stacking interface, 5′−3′ stacking interface, and a 3′−3′ stacking interface. For the 5′−5′ stacking, the structure was stable with rmsd of the G-tetrad core of 0.79 ± 0.16 Å. For the 3′−5′ and the 3′−3′ stacking, the simulations were found to be stable with rmsd of the G-tetrad core of 0.58± 0.08 and 0.53 ± 0.07 Å, respectively. We characterize the stacking between adenines across the interface of two blocks by the distance between the center of mass of the two bases and the angle between the normal vector of one base and the plane of the second base; thus, an angle of 90° corresponds to the bases being parallel.

RESULTS AND DISCUSSION

Four and Eight UUAGGG Repeats Form Abundant and Stable Structural Units in Long Human TERRA. It was reported that RNA G-quadruplexes resist the cleavage activity of RNase T1,[20,22] an endoribonuclease that cuts RNA after an unpaired guanine. It was also shown that RNase T1 cleavage of long human TERRA[22] generated products of discrete lengths matching multiples of ∼24 nt, which corresponds to four UUAGGG repeats, the minimal TERRA fragment required to form an intramolecular three-layered G-quadruplex, henceforth designated as a single G-quadruplex block.

In this work, long human TERRA was subjected to a broad range of RNase T1 cleavage extent, and the products were characterized against synthetic TERRA fragments of known sizes. A 96-repeat human TERRA sequence produced by *in vitro* transcription[22] was incubated in K^+ solution and was subsequently treated with different concentrations of RNase T1 at 25 °C for 1 h. The samples were then denatured and analyzed on 8 M urea denaturing polyacrylamide gel electrophoresis (PAGE) (Figure 1a). The three lanes on the left show the migration of three synthetic reference markers (lane 1: 12-nt 2-repeat r[UAGGGUUAGGGU]; lane 2: 23-nt 4-repeat r[UAGGG(UUAGGG)$_3$]; lane 3: 51-nt 8-repeat r[(UUAGGG)$_8$UUA]. Lane 4 corresponds to undigested long TERRA, while lanes 5−10 show digested products with increasing concentration of RNase T1 from lane 10 to lane 5. Consistent with previous observations,[22] discrete major bands were present for digested TERRA. Up to eight major bands could be seen on the lanes with low RNase T1 concentrations. The two fastest-migrating bands at the bottom of the gel were the most intense. With the increasing concentration of RNase T1, the intensities of the slower migrating bands at the top diminished, while those of the two fastest migrating bands at the bottom were nearly unchanged or even enriched (Figure 1b). The ratio between the two latter bands varied somewhat from one experiment to another (data not shown). The migration rates of these two bands were comparable to those of the synthetic 23-nt (lane 2) and 51-nt (lane 3) human TERRA reference sequences (Figure 1a), indicating that they correspond to four and eight repeats, respectively. These bands were also resistant against RNase T1 cleavage in experiments with variable digestion times (data not shown) and were present upon digestion of a 9-repeat human TERRA construct (Figures S1 and S2, Supporting Information). Similar results were obtained when the RNA transcript was annealed or simply incubated in K^+ solution at room temperature (Figure S3, Supporting Information). Introduction of a G-to-A mutation in each repeat, which transformed UUAGGG repeats to UUAGAG repeats (Figure S1, Supporting Information) and disrupted G-tract sequences, completely abolished the discrete RNase T1 cleavage protection pattern (Figure S4, Supporting Information). These findings suggested that G-quadruplexes formed by four and eight human telomeric repeats are abundant and stable structural units in long human TERRA. For intramolecular folding of a long TERRA molecule, these would likely correspond to structures containing one and two G-quadruplex block(s), respectively.

It should be noted that a previous study of RNase T1 cleavage of long TERRA[22] indicated the existence of a very intense band. This was also observed in the current study at low RNase T1 concentrations. We could demonstrate using various RNA constructs that this band contains mainly a 5′-end fragment of the transcript, which is an experimental artifact and is not related to human telomeric repeats (Figures S1 and S2, Supporting Information).

Along with the major bands, discrete minor bands (three between each pair of major bands) were present here upon digestion of TERRA, which were not observed previously.[22] These should correspond to one or multiple four-repeat block(s) plus one, two, or three UUAGGG repeat(s). Several possible reasons might account for their presence. Successive G-quadruplex blocks might have been separated by several UUAGGG repeats, or there could have been G-quadruplexes with long loops spanning one or more repeats. In addition, the G-quadruplexes are dynamic and might have undergone partial unfolding, rendering the strands susceptible to RNase cleavage. Furthermore, a part, or even the whole, of some TERRA molecules might not have adopted G-quadruplexes but other as yet unknown structures.

Formation of Parallel-Stranded G-Quadruplexes by Human TERRA in K^+ Solution. We recorded ^{1}H NMR and CD spectra of the 8-repeat human TERRA sequence r[(UUAGGG)$_8$UUA] in K^+ solution (Figure 2). The observation of sharp imino proton peaks at 10−12 ppm indicated the formation of G-quadruplexes. The CD spectra showed a negative peak at 240 nm and a positive peak at 260 nm, characteristic of parallel-stranded G-quadruplexes.[45] Previous structural studies,[19−28] combined with the current

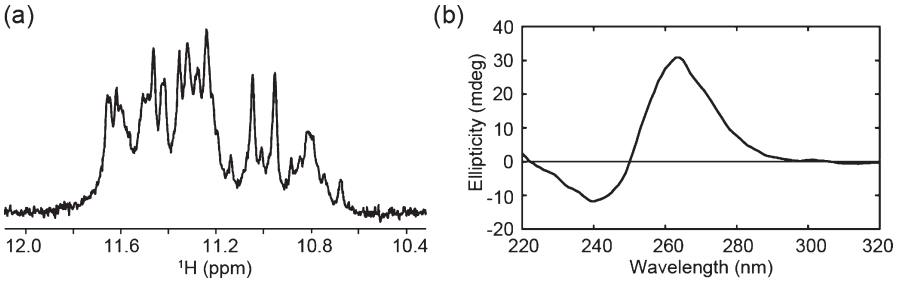

Figure 2. (a) Imino proton NMR and (b) CD spectra of the 8-repeat human TERRA sequence r[(UUAGGG)$_8$UUA] in K^+ solution at 35 °C. RNA strand concentration was 50 μM for (a) and 2 μM for (b).

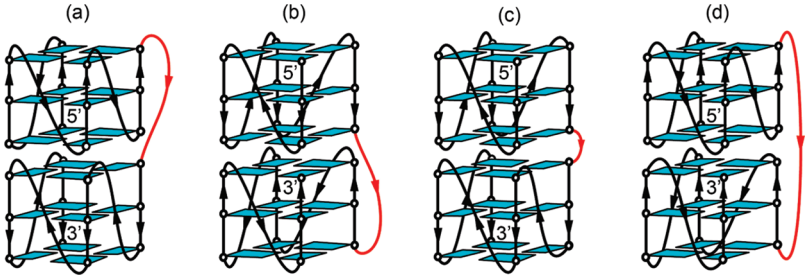

Figure 3. Representative schematics of the four possible modes of stacking for two consecutive G-quadruplex blocks connected by a UUA linker (colored in red): (a) 5′−5′, (b) 3′−3′, (c) 3′−5′, and (d) 5′−3′.

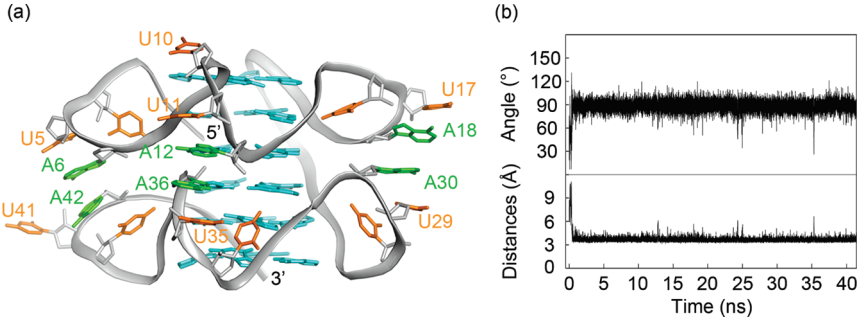

Figure 4. MD simulation results of the 5′−5′ stacking arrangement of two consecutive G-quadruplex blocks connected by a UUA linker. (a) A snapshot showing loop−loop interactions between the two G-quadruplex blocks. Note the continuous stacking between the pairs of bases A6-A42, A12-A36, and A18-A30. Guanine, adenine, and thymine bases are colored cyan, green, and orange, respectively. (b) Graphs showing the angle between the normal to base A12 and the plane of base A36 (top) and the distance between their centers of mass (bottom) over the simulation time.

ribonuclease protection, NMR, and CD data, suggested the formation of successive parallel-stranded G-quadruplexes along human TERRA.

Solvent exchange ($H_2O \rightarrow D_2O$) experiment on the 8-repeat human TERRA sequence r[(UUAGGG)$_8$UUA] in K^+ solution showed high degree of imino proton protection, supporting the formation of G-quadruplexes. However, the spectral overlap and the existence of multiple G-quadruplex conformations including aggregations prevented us from further structural interpretations.

The thermal stability of 4-, 8-, and 9-repeat human TERRA sequences in K^+ solution was characterized in UV melting experiments. In solution containing 20 mM potassium phosphate (pH 7) and 50 mM KCl, the melting temperature (T_m) of the r[UAGGG(UUAGGG)$_3$UU], r[(UUAGGG)$_8$UUA], and r[(UUAGGG)$_9$UUA] sequences was 77.1 ± 0.9, 75.6 ± 1.1, and 72.2 ± 1.0 °C, respectively (Table S1, Supporting Information). Thus, only a small difference (~1.5 °C) was observed between the T_m values of the 4- and 8-repeat human telomeric RNA sequences, in contrast to a larger difference (~5.6 °C) reported for the DNA counterparts.[32,33]

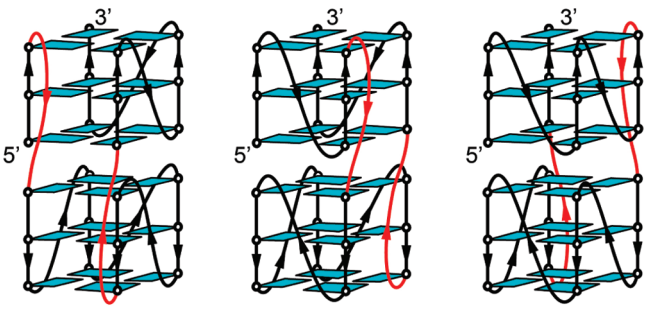

Figure 5. Representative schematics of three possible 5′−5′ stacking arrangements of two G-quadruplex blocks connected by two linkers (colored in red).

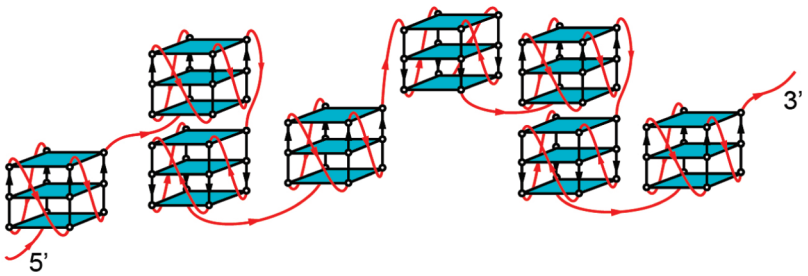

Figure 6. "Beads-on-a-string" model of long human TERRA. One and two blocks of three-layered G-quadruplexes serve as structural units along the molecule. Each parallelogram represents one layer of G-tetrad.

Stacking of Two G-Quadruplex Blocks in 8-Repeat Human TERRA Sequence: Models and Molecular Dynamics Simulations. The identification of two blocks of three-layered G-quadruplexes as a stable structural unit in long TERRA is consistent with previous reports on the stacking of parallel-stranded DNA and RNA G-quadruplexes.[21,25,26] The stacking of two blocks of three-layered parallel-stranded G-quadruplexes have been observed for human telomeric RNA sequences by gel shift,[21,26] NMR,[21] X-ray crystallography,[25] and mass spectrometry.[26]

In principle, an eight-repeat human TERRA sequence can stack as two consecutive G-quadruplex blocks, connected by a UUA linker, in four different arrangements (Figure 3): (i) 5′-to-5′, in which the stacking interface is formed by the 5′-end of each block; (ii) 3′-to-3′, in which the stacking interface is formed by the 3′-end of each block; (iii) 3′-to-5′, in which the stacking interface is formed between the 3′-end of the first block and the 5′-end of the second block; and (iv) 5′-to-3′, in which the stacking interface is formed between the 5′-end of the first block and the 3′-end of the second block. For each of these arrangements, a range of rotational isomers are possible, confined by the length of the UUA linker between the two blocks.

We performed MD simulations of the four different stacking modes (with different relative orientations between the two blocks) to test their feasibility. The starting structure was built from one block of parallel-stranded TERRA G-quadruplex taken from a crystal structure (PDB ID: 3IBK)[25] or a NMR structure (PDB ID: 2KBP).[21] The simulations were performed for 42 ns with snapshots taken every 1 ps (see Materials and Methods and Supporting Information). The 5′−5′, 3′−3′, and 3′−5′ stacking conformations were stable throughout the course of simulations, whereas the 5′−3′ stacking mode was found to be unstable, as a 3-nt linker has to stretch considerably to span six G-tetrad layers. Continuous stacking of the loop bases and extensive loop−loop interactions, particularly adenine−adenine stacking across the two blocks, were observed for the 5′−5′ stacking arrangement (Figure 4; Figure S5, Supporting Information), consistent with previous observations of NOEs between loop residues from two G-quadruplex blocks, formed by a short human telomeric RNA sequence, that are also stacked via the 5′−5′ interface.[21] This continuous stacking of loop bases was not observed in the simulations of other stacking arrangements. The stacking of the two G-tetrads at the interface showed partial overlap of the five- and six-membered rings of the guanine bases (Figure S6, Supporting Information).

Possible Structures of Two G-Quadruplex Blocks Connected by More Than One Linker. Aside from the aforementioned arrangements involving tandemly stacked G-quadruplex blocks connected by one UUA linker (Figure 3), an eight-repeat TERRA sequence can also fold back-and-forth between the two G-quadruplex blocks, such that the two blocks are connected by two to seven linkers, akin to a dimeric G-quadruplex formed by the *c-kit2* promoter sequence.[46] Models for the 5′−5′ stacking arrangement consisting of two linkers (shown schematically in Figure 5) were generated and our first MD studies suggested that they are stable (data not shown). Structures consisting of three or more linkers are also topologically possible but might be constrained by some linker lengths; i.e., a 3-nt UUA linker is too short for some connections.

CONCLUSION

Our data have suggested that G-quadruplex stacks containing either one or two blocks of three-layered G-quadruplex(es), formed by four- and eight-repeat sequences, respectively, are stable structural units in long human TERRA. Various stacking possibilities exist for the structures of the eight-repeat sequence. MD simulations suggested that the structure with 5′−5′ stacking of two blocks could provide continuous stacking of the loop bases as well as loop−loop interactions between the two subunits. We hereby propose a structural model for long human TERRA, in which G-quadruplexes are arranged as "beads-on-a-string",[30,33] where each bead consists of either a single block of three-layered G-quadruplex or two blocks of three-layered G-quadruplexes that are stacked atop one another (Figure 6). Note however that this does not preclude the possibility of further stacking of the G-quadruplex blocks into higher-order assemblies,[21,26,29] which might occur partially or transiently along TERRA, or possible associations of multiple TERRA molecules. Our results will be useful for the design of drugs targeted to G-quadruplexes formed in long telomeric sequences.[47]

ASSOCIATED CONTENT

Supporting Information. Table S1 showing melting temperature of various human TERRA sequences; Figures S1−S6 showing plasmid and DNA template constructs, denaturing PAGE, and results of MD simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

TERRA, telomeric RNA; MD, molecular dynamics; NMR, nuclear magnetic resonance; CD, circular dichroism; UV, ultraviolet; rmsd, root-mean-square deviation; PAGE, polyacrylamide gel electrophoresis; PDB, Protein Databank.

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