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<th>Structure of the human telomere in Na+ solution : an antiparallel (2+2) G-quadruplex scaffold reveals additional diversity</th>
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<td>Author(s)</td>
<td>Lim, Kah Wai; Ng, Veronica Chinn Min; Martin-Pintado, Nerea; Heddi, Brahim; Phan, Anh Tuan</td>
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Structure of the human telomere in Na\textsuperscript{+} solution: an antiparallel (2+2) G-quadruplex scaffold reveals additional diversity

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ABSTRACT

Single-stranded DNA overhangs at the ends of human telomeric repeats are capable of adopting four-stranded G-quadruplex structures, which could serve as potential anticancer targets. Out of the five reported intramolecular human telomeric G-quadruplex structures, four were formed in the presence of K\textsuperscript{+} ions and only one in the presence of Na\textsuperscript{+} ions, leading often to a perception that this structural polymorphism occurs exclusively in the presence of K\textsuperscript{+} but not Na\textsuperscript{+}. Here we present the structure of a new antiparallel (2+2) G-quadruplex formed by a derivative of a 27-nt human telomeric sequence in Na\textsuperscript{+} solution, which comprises a novel core arrangement distinct from the known topologies. This structure complements the previously elucidated basket-type human telomeric G-quadruplex to serve as reference structures in Na\textsuperscript{+}-containing environment. These structures, together with the coexistence of other conformations in Na\textsuperscript{+} solution as observed by nuclear magnetic resonance spectroscopy, establish the polymorphic nature of human telomeric repeats beyond the influence of K\textsuperscript{+} ions.

INTRODUCTION

Telomeres, the ends of linear eukaryotic chromosomes, perform vital roles in the maintenance of genome integrity and the regulation of cell proliferation (1). Human telomeric DNA consists of thousands of tandem repeats of (TTAGGG)\textsubscript{n} sequences (2,3), terminating as single-stranded DNA of 100–200 nt at the 3' ends (4). Such guanine-rich repeats are capable of adopting four-stranded G-quadruplex structures (5–8) under physiological conditions. G-quadruplex formation at the telomeric ends has been shown to inhibit the activity of the enzyme telomerase (9), which is essential for the proliferation of most cancer cells (10,11). Insights from high-resolution G-quadruplex structures adopted by these sequences could thus aid in drug design efforts targeting telomeric DNA for potential anticancer treatment (12,13).

To date, at least five distinct intramolecular G-quadruplex folding topologies (Supplementary Figure S1) have been reported for natural human telomeric repeats (14–23), four of which were observed in the presence of K\textsuperscript{+} ions. Form 1 (15–17) and Form 2 (18,19), observed in K\textsuperscript{+} solution, both comprise the (3+1) G-tetrad core, but differ in procession of the different loops; Form 3 (20), also observed in K\textsuperscript{+} solution, consists of a two-G-tetrad basket-type core with extensive base stacking interactions in the loops. In a K\textsuperscript{+}-containing crystal (21) and under water-depleted conditions (22,24–26), the all-parallel-stranded propeller-type G-quadruplex structure was shown to be the preferred form. On the other hand, the only conformation to have been characterized in Na\textsuperscript{+} solution consists of a three-layered basket-type G-quadruplex (23). Myriad studies on human telomeric G-quadruplexes have been carried out using a wide variety of techniques (27–41), ranging from ultraviolet (UV) (27), circular dichroism (CD) (28), fluorescence (29–31), Raman (32), electron paramagnetic resonance (33) and nuclear magnetic resonance (NMR) spectroscopy (34), to X-ray crystallography (21), mass spectrometry (29–31), Raman (32), electron paramagnetic resonance (33) and nuclear magnetic resonance (NMR) spectroscopy (34), to X-ray crystallography (21), mass spectrometry (35), gel electrophoresis (36), single-molecule Förster resonance energy transfer (37,38), laser-tweezers pulling (39), calorimetry (40) and molecular dynamics (MD) simulations (41), and the results have revealed the polymorphic nature of human telomeric repeats in the presence of K\textsuperscript{+}
ions. While not fully addressed, this structural polymorphism has also been implicated in Na⁺ solution (29,36,37), yet the basket form has largely been used as the sole reference structure for interpretation of studies under Na⁺-containing environment. Knowledge on the structural disparities, if any, in the presence of Na⁺ ions would contribute toward fundamental understanding of the cationic influence on human telomeric repeats under different contexts. Using NMR, here we show that four-repeat human telomeric sequences could adopt multiple G-quadruplex conformations in Na⁺ solution. We present the solution structure of one of these forms, which exhibits features distinct from the known topologies. This latest addition to the ensemble of telomeric G-quadruplex structures establishes the polymorphic nature of human telomeric repeats beyond the influence of K⁺ ions, and builds on the available pool of structural motifs for targeting of these important entities.

METHODS

Sample preparation

Unlabeled (Supplementary Table S1) and site-specific labeled (2%-15N-enriched or ²H-labeled; Supplementary Table S2) DNA oligonucleotides were chemically synthesized on an ABI 394 DNA/RNA synthesizer using products from Glen Research, Spectra Gases and Cambridge Isotope Laboratories. The oligonucleotides were de-protected following the manufacturer’s protocols and purified using Poly-Pak™ cartridges. Samples were dialyzed successively against water, ~25 mM NaCl solution and water again. They were subsequently frozen, lyophilized and suspended in a buffer containing 20 mM sodium phosphate (pH 7.0) and 70 mM NaCl. DNA concentration is expressed in strand molarity using a nearest-neighbor approximation for the absorption coefficients of the unfolded species (42). The same extinction coefficient was used for the natural and ¹³⁵G-substituted oligonucleotides.

UV spectroscopy

Thermal stability of different DNA oligonucleotides was characterized by recording the UV absorbance at 295 nm (43) as a function of temperature (20–90°C) using a JASCO V-650 spectrophotometer. The heating and cooling rates were 0.2°C/min. Two baselines corresponding to the completely folded (low temperature) and completely unfolded (high temperature) states were manually drawn to determine the fractions of folded and unfolded species during the melting/folding transition. The melting temperature (T_m) is defined as the temperature for which there are equal fractions of folded and unfolded species. Experiments were performed with quartz cuvettes (1-cm pathlength for low DNA concentrations and 0.2-cm pathlength for high DNA concentrations).

CD spectroscopy

CD spectra were recorded at 20°C on a JASCO-815 spectropolarimeter over the range of 220–320 nm using a 1-cm pathlength quartz cuvette with a reaction volume of 600 µL. For each sample, an average of three scans was taken, the spectrum of the buffer was subtracted and the data were zero-corrected at 320 nm.

NMR spectroscopy

Strand concentration of the NMR samples was typically 0.5–2.0 mM. NMR experiments were performed on Bruker AVANCE 600- and 700-MHz spectrometers at 25°C, unless otherwise specified. Resonances for guanine residues were assigned unambiguously using site-specific low-enrichment ¹⁵N labeling (44), site-specific ²H labeling (45) and through-bond correlations at natural abundance (46,47). Spectral assignments were assisted by NOESY, COSY, TOCSY and [¹H,¹³C]-HSQC experiments. The spectra were processed with the software TopSpin and analyzed using the program FELIX (Felix NMR, Inc.).

Structure calculation

Inter-proton distances for the d[(TTAGGG)₃TTA(¹³⁵G)GGTTA] (htel27) G-quadruplex were deduced from NOESY experiments performed in H₂O (mixing time, 200 ms) and D₂O (mixing times, 100 and 350 ms). Structures were calculated based on distance-restrained molecular dynamics refinement following distance geometry simulated annealing using the program XPLOR-NIH (48). Hydrogen bond restraints, inter-proton distance restraints, dihedral restraints, planarity restraints and repulsive restraints were imposed during structure calculations. Structures were displayed using the program PyMOL (49). Detailed procedures for structure calculation are described in Supplementary Text.

Data deposition

The coordinates for the d[(TTAGGG)₃TTA(¹³⁵G)GGTTA] (htel27) G-quadruplex have been deposited in the Protein Data Bank (access code 2MBJ).

RESULTS AND DISCUSSION

Four-repeat human telomeric sequences adopt diverse G-quadruplex conformations in Na⁺ solution

NMR spectra of four-repeat natural human telomeric sequences with different flanking ends (Supplementary Table S1) in Na⁺ solution indicated the adoption of multiple conformations (Supplementary Figure S2). In agreement with the previous study (23), the sequence d[AGGG(TTAGGG)]₃ (denoted as htel22; Table 1) displayed 12 major imino proton peaks at ~10.6–12.0 ppm (Figure 1A), corresponding to the establishment of the basket-type G-quadruplex (Supplementary Figure S1A). In addition, two sequences with the same 5’-flanking end as htel22 showed a major species with a highly similar spread of imino proton peaks (Supplementary Figure S2F and G), suggesting their adoption of the same topology as the prevailing form. The other sequences mostly displayed the coexistence of two or more major species, judging from the number and intensity of imino.
proton peaks. CD spectra of all 16 sequences displayed two positive maxima at ~250 and ~295 nm, and a trough at ~260–270 nm (Supplementary Figure S3). The 295-nm peak is characteristic of opposite-polarity stacking of G-tetrads (50), suggesting that these sequences largely conform to antiparallel G-quadruplexes in Na⁺ solution. Note that h tel22 produced a sharp negative trough at ~260 nm (Figure 1B), which was less intense in the other sequences.

Formation of a G-quadruplex with an alternative conformation distinct from h tel22

We chose to probe the structures present in the sequence d[(TTAGGG)₄TTA] (denoted as h tel27; Table 1) (Figure 1A), which has the largest number of flanking nucleotides for potential interactions. Previously, 8-bromoguanine (BrG)-for-G substitution has been used to drive the folding of an oligonucleotide toward a single major G-quadruplex structure by favoring the syn glycosidic conformation at the substituted residue (17,18,20,51). Here, single BrG incorporation at position G22 of h tel27, giving rise to the sequence d[(TTAGGG)₃TTA(BrG)GGTTA] (denoted as h tel27[Br22]; Table 1), led to the emergence of a predominant species (>85%) amenable for detailed structural characterization (Figure 1A). The guanine imino and H8 proton assignments were further corroborated by through-bond correlations at natural abundance (46) (Supplementary Figure S5) was completed with the assistance of other through-bond correlation experiments (COSY, TOCSY and {¹H-¹³C}-HSQC; data not shown) (47). The strong intensity of intraresidue H8-H1 NOE cross-peaks for G4, G5, G10, G16 and G23 (Supplementary Figure S6) indicated that these guanine residues, together with BrG22, adopt the syn glycosidic conformation. All other guanine residues adopt the anti glycosidic conformation.

H tel27[Br22] adopts a new antiparallel (2+2) folding topology in Na⁺ solution

Having determined the guanine imino and H8 proton assignments for h tel27[Br22], the alignment of the tetrads could then be deduced based on characteristic cyclic imino–H8 NOE connectivity patterns within each tetrad (Figure 3A and B). The top G-tetrad (G6•G24•G16•G10) were closely related. Their CD spectra were substantially different from that of h tel22, notably the absence of the sharp negative trough at ~260 nm, pointing to the adoption of an alternative folding topology. Melting analyses of the three sequences were carried out by monitoring the UV absorbance at 295 nm (43), and the melting temperatures (Tm) of h tel22, h tel27 and h tel27[Br22] were found to be 57.6, 44.5 and 50.8°C, respectively, in ~100 mM Na⁺ (Supplementary Figure S4). The BrG-substituted oligonucleotide displayed a $T_m$ ~6°C higher than that of the natural counterpart, consistent with observations from previous studies (20,51).

NMR spectral assignments

To proceed with the structural elucidation of h tel27[Br22], guanine imino (Figure 2A) and adenine/guanine H8 (Figure 2B) protons were unambiguously assigned using site-specific low-enrichment ¹⁵N labeling (44) and site-specific ²H labeling (45), respectively (Supplementary Table S2). The guanine imino and H8 proton assignments were further corroborated by through-bond correlations at natural abundance (46) (Figure 2C). Assignments of thymine residues were supplemented by T-to-U substitutions (Supplementary Table S2). The H8/H6-H1' NOE sequential connectivity of h tel27[Br22] (Supplementary Figure S5) was completed with the assistance of other through-bond correlation experiments (COSY, TOCSY and [¹H,¹³C]-HSQC; data not shown) (47). The strong intensity of intraresidue H8-H1' NOE cross-peaks for G4, G5, G10, G16 and G23 (Supplementary Figure S6) indicated that these guanine residues, together with BrG22, adopt the syn glycosidic conformation. All other guanine residues adopt the anti glycosidic conformation.

Table 1. Representative natural and modified human telomeric sequences used in this study

<table>
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<th>Sequence</th>
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<tr>
<td>h tel22</td>
<td>A GGG TTA GGG TTA GGG TTA GGG</td>
</tr>
<tr>
<td>h tel27</td>
<td>TTA GGG TTA GGG TTA GGG TTA GGG TTA</td>
</tr>
<tr>
<td>h tel27[Br22]</td>
<td>TTA GGG TTA GGG TTA GGG TTA GGG TTA BrG</td>
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*The modified 8-bromoguanine (BrG) residue is in boldface.
is oriented in the opposite hydrogen-bond directionality with respect to the other two G-tetrads, (G5\(\rightarrow\)C15\(\rightarrow\)G11\(\rightarrow\)C15\(\rightarrow\)G17\(\rightarrow\)C15\(\rightarrow\)G23) and (G4\(\rightarrow\)C15\(\rightarrow\)G12\(\rightarrow\)C15\(\rightarrow\)G18\(\rightarrow\)C15\(\rightarrow\)BrG22), and the glycosidic conformations of guanines are ant/anti/syn for all three tetrads. The central positioning of (G5\(\rightarrow\)C15\(\rightarrow\)G11\(\rightarrow\)C15\(\rightarrow\)G17\(\rightarrow\)C15\(\rightarrow\)G23) in the tetrad core agrees with solvent exchange data showing that the imino protons from this G-tetrad were the most protected (Supplementary Figure S7). Connecting the sequential guanine residues and joining the corners of the G-tetrad core with the linking TTA loops, we derived an antiparallel G-quadruplex scaffold consisting of two adjacent strands pointing up and two remaining strands pointing down (up–up–down–down) or a (2+2) G-quadruplex scaffold (14) with a successive loop arrangement edgewise–propeller–edgewise (Figure 3C).

**Na\(^+\) solution structure of the htel27{Br22} G-quadruplex**

The structure of htel27{Br22} G-quadruplex in Na\(^+\) solution (Figure 4) was computed on the basis of NMR restraints (Table 2). There are one wide, one narrow and two medium grooves. The first (edgewise) loop crosses the top of the narrow groove, the second (propeller) loop traverses across one of the medium grooves, while the third (edgewise) loop completes a turn over the bottom of the wide groove. The 5'- and 3'-flanking TTA nucleotides take part in hydrogen-bond and stacking interactions at the bottom and the top of the structure, respectively. There is a Watson–Crick A9\(\rightarrow\)T25 base pair capping the top of the tetrad core. A15 from the propeller loop also stacks over the top G-tetrad, potentially establishing interactions with the loop elements at the top. T14 from the same loop projects into the medium groove, whereas T13 flips out of the propeller loop. At the bottom of the structure, A3 and T20 establishes a Watson–Crick base pair, with A21 completing the formation of a triple platform. These stabilizing interactions of the loop and terminal residues would agree with the observation of additional broad imino proton peaks at ∼12.2–14.0 ppm (Supplementary Figure S8). The establishment of base pair stacking interactions across the ends of the tetrad core could have provided considerable contribution toward the adoption of the major conformation of htel27{Br22}, consistent with previous observations regarding other human telomeric G-quadruplex structures formed in K\(^+\) solution (15–20). In a longer sequence context, the various structural forms could exist in a state of equilibrium, with possible interconversion among them.
Novel features of the htel27[Br22] G-quadruplex

We have shown that in Na⁺ solution, htel27[Br22] assumes a novel antiparallel (2+2) folding topology (Figure 3C). Although the tetrad cores of htel27[Br22] (this work) and htel22 (23) possess the same relative strand orientations, they differ in the hydrogen-bond directionalities of the tetrads: anticlockwise–clockwise–clockwise for htel27[Br22] versus clockwise–anticlockwise–clockwise for htel22 (Supplementary Figure S9). Loop arrangement of htel27[Br22] (edgewise–propeller–edgewise) is also different from that of htel22 (edgewise–diagonal–edgewise). To date, antiparallel (2+2) G-quadruplexes that have been investigated largely consist of edgewise and/or diagonal loops (20,23–54), while quadruplexes containing propeller loops mostly belong to the all-parallel-stranded (21,22) or (3+1) (15–19,55–57) core topology. Antiparallel (2+2) G-quadruplexes with a propeller loop (58), such as that formed by htel27[Br22], have not been observed in the context of human telomeric repeats.

CONCLUSION

The antiparallel (2+2) G-quadruplex scaffold adopted by htel27[Br22] (and presumably a subpopulation of htel27), as well as the observation of the coexistence of multiple conformations in the series of four-repeat natural human telomeric sequences in Na⁺ solution, point to additional diversity than had been previously assumed. Even though intracellular Na⁺ content is ~10 times lower than that of K⁺, these structures observed in Na⁺ solution could potentially take part in the interconversion between various quadruplex forms.

Table 2. Statistics of the computed structures of the 27-nt BR-G-modified human telomeric sequence d[(TTAGGG)₃TTA(BrG)GGTTA] (htel27[Br22])

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<tr>
<td>Intraresidue distance restraints</td>
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<td>Sequential (i, i+1) distance restraints</td>
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<tr>
<td>Long-range (i, i+2) distance restraints</td>
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</tr>
<tr>
<td>Other restraints</td>
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<tr>
<td>Hydrogen bond restraints</td>
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<td>Planarity restraints</td>
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<td>Repulsive restraints</td>
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B. Structure statistics for 10 molecules following distance-restrained MD refinement

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<td>Maximum violation (Å)</td>
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<tr>
<td>RMSD of violations (Å)</td>
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<tr>
<td>Deviations from the ideal covalent geometry</td>
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<tr>
<td>Bond lengths (Å)</td>
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<td>Bond angles (°)</td>
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<tr>
<td>Improper (°)</td>
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<tr>
<td>Pairwise all heavy atom RMSD values (Å)</td>
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<td>1.49 ± 0.35</td>
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*PDB ID: 2MBJ.

**Distance restraints between pairs of protons that do not exhibit NOE cross-peaks.
ACCESSION NUMBER

PDB: accession code 2MBJ.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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