

# Mechanical unfolding of long human telomeric RNA (TERRA)<sup>†</sup>

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**We report the first single molecule investigation of TERRA molecules. By using optical-tweezers and other biophysical techniques, we have found that long RNA constructions of up to 25 GGGUUA repeats form higher order structures comprised of single parallel G-quadruplex blocks, which unfold at lower forces than their DNA counterparts.**

Telomeres are nucleoprotein structures that protect chromosome ends from being recognized as DNA breaks.<sup>1</sup> Mammalian telomere DNA consists of long tandem arrays of double-stranded TTAGGG repeats that are maintained by the telomerase.<sup>2</sup> The 3' end of each telomere terminates in a G-rich single-stranded overhang (150–200 nucleotides) that is able to self-fold into G-quadruplexes. The demonstration of the *in vivo* presence of DNA G-quadruplexes at telomeres suggests that these non-canonical secondary structures have a role in telomere end-protection, and therefore in chromosome stability.<sup>3,4</sup> It has also been found that telomere end-binding proteins control the folding and unfolding of DNA G-quadruplexes *in vitro*<sup>5</sup> and *in vivo*.<sup>4</sup>

Telomeres are transcribed from the subtelomeric regions towards chromosome ends into telomeric repeat-containing RNA (TERRA).<sup>6,7</sup> These non-coding RNA molecules contain subtelomere-derived sequences and an average of 34 GGGUUA repeats at their 3' end.<sup>8</sup> TERRA acts as a scaffold for the assembly of telomeric proteins involved in telomere maintenance and telomeric heterochromatin formation.<sup>9,10</sup> Importantly, there is also evidence for the presence of TERRA RNA G-quadruplexes in living cells.<sup>11</sup>

It has been shown by nuclear magnetic resonance (NMR) spectroscopy<sup>12,13</sup> and by X-ray crystallography<sup>14</sup> that short TERRA molecules form parallel-stranded G-quadruplex structures but high-resolution structures of long telomeric RNA have not yet been obtained. Electrospray mass spectrometry<sup>15</sup> and computational analysis<sup>12,16</sup> of long TERRA molecules have suggested stacking between their quadruplexes. The largest TERRA molecule (51nt) analyzed by NMR has shown intramolecular parallel G-quadruplexes.<sup>16</sup>

Here, we report the synthesis and purification of TERRA molecules up to 96nt (twice the largest molecule previously analyzed by NMR), a size similar to the endogenous nuclear TERRA<sup>8</sup> (see ESI<sup>†</sup>). We have also used optical-tweezers (OT) to study RNA molecules with different numbers of possible quadruplexes ranging from 1 (Q1) to 6 (Q6).

CD spectra of the RNA sequence Q4 in 25 mM potassium phosphate pH 7 buffer showed a positive band at 260 nm and a negative band at 240 nm (Fig. 1a), the characteristic signature of parallel-stranded G-quadruplex conformations. This result is in agreement with previously reported observations showing that other telomeric RNA sequences are folded into parallel G-quadruplex structures both in sodium and potassium solutions.<sup>12,13,16–20</sup> It is also important to mention that no band at 300 nm was observed for this long telomeric RNA as it was reported for 22-mer and 45-mer telomeric RNA in ammonium acetate buffer.<sup>15</sup> On the other hand, the normalized CD spectra of the bimolecular G-quadruplex formed by r(UAGGGUUAGGGU) and Q4 showed a similar shape and amplitude, which indicates that most of the Q4 molecules form four G-quadruplexes (Fig. 1b).<sup>21</sup>

Thermal stability of Q4 was also measured by recording CD melting curves. To test the influence of the potassium concentration on the thermal stability we performed the experiments in two different potassium-containing buffers (Fig. 1c). Melting temperatures of 65.5 °C and 72.5 °C were obtained in 25 mM potassium phosphate pH 7 and 25 mM potassium phosphate pH 7 buffer plus 50 mM KCl respectively. Such a notable difference between melting temperatures confirms the influence of potassium concentration on the thermal stability of RNA G-quadruplexes.<sup>22</sup> On the other hand, comparison of melting temperatures of Q4 and intramolecular telomeric RNA sequences reported in the

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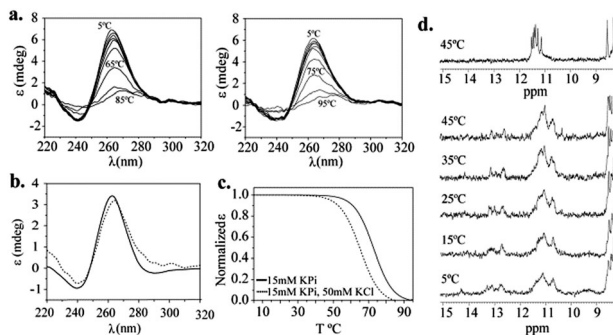
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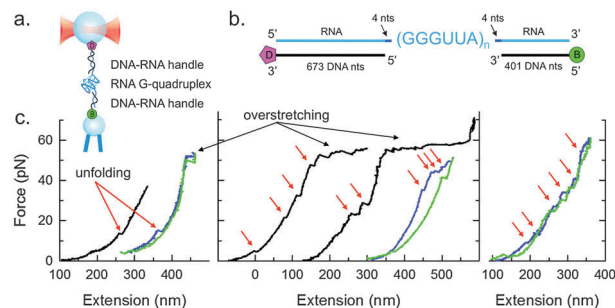
**Fig. 1** (a) Series of CD spectra of Q4 in 15 mM KPI pH 7 buffer (left) and 15 mM KPI pH 7, 50 mM KCl (right) at different temperatures, [oligonucleotide] = 2  $\mu$ M. (b) CD melting curves of Q4 at different K<sup>+</sup> concentrations. (c) CD spectra normalized for G-quadruplex concentration of r(UAGGGUUAGGGU) (straight line) and Q4 (dotted line) at 25 °C in buffer 25 mM KPI pH 7 and 15 mM KPI pH 7 respectively. (d) NMR spectra of r(UAGGGUUAGGGU) at 45 °C (top) and Q3 (15 GGGUUA repeats) at different temperatures (bottom) in H<sub>2</sub>O/D<sub>2</sub>O 9 : 1 in 15 mM KPI pH 7 buffer. RNA concentration of 100  $\mu$ M and 10  $\mu$ M, respectively.

bibliography<sup>17,18</sup> suggests that the length of the sequence is not necessarily a factor that enhances thermal stability.

Large scale synthesis and purification of long telomeric RNA were performed following previously described protocols<sup>23</sup> yielding enough amount of sample to acquire 1D H-NMR spectra. Despite the higher widening of the signals produced by the size of the molecule, the shape of the NMR spectra is comparable to the one observed for r(UAGGGUUAGGGU), showing signals between 10 and 12 ppm, the characteristic chemical shifts of guanine imino protons forming G-tetrads (Fig. 1d). Moreover, the imino proton signals remain visible at 55 °C indicating a remarkable thermostability, consistent with G-quadruplex folding. Interestingly, imino proton signals at around 13 ppm are also visible in the whole range of temperature indicating contacts involving nucleotides of the loops. To our knowledge, the presence of these signals has not been observed in other NMR studies on telomeric RNA sequences. This fact suggests the occurrence of loop-loop interactions between different quadruplexes. These interactions are consistent with A:U/T base pairs observed in the X-ray structures of the telomeric RNA<sup>14</sup> or DNA quadruplex.<sup>24</sup>

The vectors employed to produce telomeric RNA in sufficiently large amounts for NMR experiments were also used to generate the constructs required for OT analysis (Fig. 2a and ESI<sup>†</sup>). Thus, three constructions with 5 (Q1), 16 (Q4) and 25 (Q6) telomeric repeats were obtained. The telomeric sequences were sandwiched between two hybrid duplex RNA:DNA acting as handles (Fig. 2b). Single molecules were stretched with OT to forces exceeding the overstretching transition of the hybrid handles,<sup>25,26</sup> following the scheme of Fig. 2a.

Fig. 2c shows, from left to right, three series of single-molecule force-extension experiments corresponding to Q1, Q4 and Q6 sample preparations, respectively. In the left panel, two experiments, each with a different molecule, are shown. Single-unfolding events are observed in accord with what was described for DNA quadruplexes.<sup>27–29</sup> Typical rupture forces are  $15 \pm 2$  pN, below those observed for DNA quadruplexes for the experimental loading rate used here ( $22 \pm 2$  pN s<sup>-1</sup>, see ESI<sup>†</sup>).<sup>27,30</sup> Weaker stacking interactions between adjacent G-tetrad planes in RNA quadruplexes with respect to those of DNA may be responsible for the lower force needed to unfold TERRA with respect to comparable DNA quadruplex motifs.

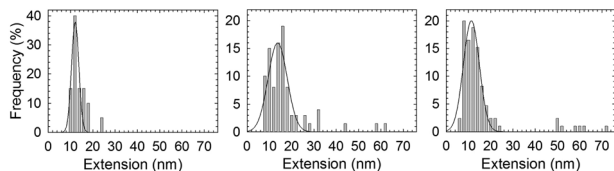


**Fig. 2** (a) Cartoon of the experiment: a Q<sub>n</sub> construction is attached between a bead (biotin–streptavidin linkage), held by suction on the top of a micropipette, and an optically trapped bead (digoxigenin–anti-digoxigenin antibody linkage).<sup>28</sup> (b) Cartoon of the OT construction showing each component and its length; biotin (B) and digoxigenin (D). (c) Force–extension curves showing unfolding events (red arrows) of Q1 (left), Q4 (middle) and Q6 (right) preparations. Black and blue traces show stretching assays and green traces are relaxation paths corresponding to the blue traces. Each stretch–relaxation curve was offset ~100 nm to increase the clarity of the figure. Black arrows mark the beginning of the overstretching transition.

A similar scenario was found for the base-stacking interactions in double-stranded (ds) nucleic acids, where the dsRNA molecules showed a lower stretch modulus in the force–extension curves with respect to their sequence-equivalent DNA counterparts.<sup>25</sup> It is also important to note that TERRA involves only parallel arrangements, which for DNA quadruplexes have been described with lower unfolding forces than anti-parallel ones.<sup>27</sup> The stretch–relaxation (blue–green) experimental curve of Fig. 2c also shows that unfolding and refolding processes in RNA G-quadruplexes are not reversible, as reflected by the fact that relaxation traces did not superimpose over the stretching paths (blue and green curves in Fig. 2c). A similar irreversibility was also previously exhibited by DNA G-quadruplexes.<sup>27</sup>

Fig. 2c, middle panel, shows three experiments in which sequential and simultaneous rupture events take place. The leftmost curve of this panel shows four sequential unfolding events, the last one taking place right before the overstretching transition of the hybrid handles, below 60 pN. The middle curve shows two unfolding events whose extension coordinate exceeds the mean unfolding distances (see below), probably due to the simultaneous opening of two G-quadruplexes.<sup>31</sup> The rightmost, blue–green experimental traces in Fig. 2c (middle) correspond to a stretch–relaxation cycle in which sequential unfolding events are almost simultaneous. The rightmost and leftmost curves of this middle panel correspond to stretch–relaxation cycles of the same molecule. This sample molecule shows additional features of the mechanical behaviour of the G-quadruplexes, namely, that G-quadruplexes do refold upon relaxation but that rupture events during a new stretching cycle do not necessarily overlap with those from previous cycles. Finally, Fig. 2c, right panel, shows a stretch–relaxation cycle of a Q6 single-molecule sample. In this case, six sequential unfolding events and their corresponding refolding ones are visible in the curves. The stretch–relaxation cycle shows, as before, a hysteretic behaviour.

RNA preparations are labile, which typically resulted in molecular breakage of the single-molecule constructs at high forces. However, some of the constructions could resist forces beyond the overstretching transition of the hybrid handles.



**Fig. 3** Unfolding extension histograms for Q1 (left,  $n = 20$ ), Q4 (center,  $n = 68$ ) and Q6 (right,  $n = 85$ ) TERRA repeats from single-molecule experiments. Peak position (and standard deviation) according to Gaussian fits to the experimental histograms are 12.1 (1.6) nm, 13.7 (4.2) nm and 11.3 (3.6) nm, respectively.

This transition provides here a control check to show that single-molecule constructs were used in the OT experiments. Analysis of this transition shows that hybrid DNA:RNA molecules approximately overstretch as A-form molecules.<sup>25</sup>

The fact that the TERRA G-quadruplex showed a clear parallel conformation is reflected in the distribution of extension change upon unfolding. Fig. 3, left panel, shows that the change in extension for Q1 preparation peaks at  $\sim 12$  nm, with no apparent mixture of populations.<sup>27,31</sup> This distance is compatible with the unfolding of one quadruplex and the presence of 14 nucleotides, distributed as one GGGUUA repeat plus 4 flanking nucleotides at both ends (see Fig. 2b and ES1<sup>†</sup>), which may interact with the RNA G-quadruplex at low force in Q1 preparation.

When several tandem quadruplexes are unfolded, the extension distribution peaks at approximately the same distance (Fig. 3, middle and right panels) but the standard deviation is larger. The conserved mean unfolding distance shows that there is a preference for sequential unfolding and the increase in standard deviation reflects that interaction between quadruplexes takes place when they are arranged in tandem repeats, as discussed above. In agreement with former literature on DNA quadruplexes,<sup>32</sup> cooperative unfolding events took place in our experiments (see Fig. 3 center and right) but were infrequent compared to sequential ones for TERRA tandem repeats. The unfolding sequence of rupture events in the force–extension traces is thus more similar to those shown for protein unfolding in tandem preparations<sup>33</sup> than other cooperative transitions in DNA or RNA (overstretching or unzipping).<sup>26</sup>

TERRA is a promising cancer therapeutic target because it is required for telomeric heterochromatin formation and because its higher-order G-quadruplex structure provides specific binding sites to optimize drug design. The single molecule characterization of TERRA molecules established here provides useful mechanistic information that can be readily used to find specific TERRA–ligand interactions, as well as in future studies on TERRA–proteins and TERRA–DNA interactions.

In conclusion, mechanical unfolding experiments of long RNA telomeric repeats (TERRA) indicate that these molecules form homogeneous parallel quadruplexes with typical rupture forces lower than their DNA counterparts. We have found that TERRA forms higher order structures at a single molecule level, which exhibit some cooperative unfolding events.

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## Notes and references

- 1 T. de Lange, *Genes Dev.*, 2005, **19**, 2100.
- 2 E. H. Blackburn, *Nature*, 1991, **350**, 569.
- 3 G. Biffi, D. Tannahill, J. McCafferty and S. Balasubramanian, *Nat. Chem.*, 2013, **5**, 182.
- 4 K. Paeschke, T. Simonsson, J. Postberg, D. Rhodes and H. J. Lipps, *Nat. Struct. Mol. Biol.*, 2005, **12**, 847.
- 5 H. Hwang, N. Buncher, P. L. Opresko and S. Myong, *Structure*, 2012, **20**, 1872.
- 6 C. M. Azzalin, P. Reichenbach, L. Khoriauli, E. Giulotto and J. Lingner, *Science*, 2007, **318**, 798.
- 7 S. Schoeftner and M. A. Blasco, *Nat. Cell Biol.*, 2008, **10**, 228.
- 8 A. Porro, S. Feuerhahn, P. Reichenbach and J. Lingner, *Mol. Cell Biol.*, 2010, **30**, 4808.
- 9 Z. Deng, J. Norseen, A. Wiedmer, H. Riethman and P. M. Lieberman, *Mol. Cell*, 2009, **35**, 403.
- 10 I. L. de Silanes, M. S. d'Alcontres and M. A. Blasco, *Nat. Commun.*, 2010, **1**, 33.
- 11 Y. Xu, Y. Suzuki, K. Ito and M. Komiyama, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 14579.
- 12 H. Martadinata and A. T. Phan, *J. Am. Chem. Soc.*, 2009, **131**, 2570.
- 13 Y. Xu, K. Kaminaga and M. Komiyama, *J. Am. Chem. Soc.*, 2008, **130**, 11179.
- 14 G. W. Collie, S. M. Haider, S. Neidle and G. N. Parkinson, *Nucleic Acids Res.*, 2010, **38**, 5569.
- 15 G. W. Collie, G. N. Parkinson, S. Neidle, F. Rosu, E. De Pauw and V. Gabelica, *J. Am. Chem. Soc.*, 2010, **132**, 9328.
- 16 H. Martadinata, B. Heddi, K. W. Lim and A. T. Phan, *Biochemistry*, 2011, **50**, 6455.
- 17 A. Arora and S. Maiti, *J. Phys. Chem. B*, 2009, **113**, 10515.
- 18 A. Joachimi, A. Benz and J. S. Hartig, *Bioorg. Med. Chem.*, 2009, **17**, 6811.
- 19 J. Qi and R. H. Shafer, *Biochemistry*, 2007, **46**, 7599.
- 20 A. Randall and J. D. Griffith, *J. Biol. Chem.*, 2009, **284**, 13980.
- 21 L. Petraccone, in *Quadruplex Nucleic Acids*, ed. J. B. Chaires and D. Graves, Springer, Berlin, Heidelberg, 2013, vol. 330, p. 23.
- 22 S. Kumari, A. Bugaut and S. Balasubramanian, *Biochemistry*, 2008, **47**, 12664.
- 23 S. A. McKenna, I. Kim, E. V. Puglisi, D. A. Lindhout, C. E. Aitken, R. A. Marshall and J. D. Puglisi, *Nat. Protoc.*, 2007, **2**, 3270.
- 24 G. N. Parkinson, M. P. Lee and S. Neidle, *Nature*, 2002, **417**, 876.
- 25 E. Herrero-Galan, M. E. Fuentes-Perez, C. Carrasco, J. M. Valpuesta, J. L. Carrascosa, F. Moreno-Herrero and J. R. Arias-Gonzalez, *J. Am. Chem. Soc.*, 2013, **135**, 122.
- 26 C. Bustamante, Z. Bryant and S. B. Smith, *Nature*, 2003, **421**, 423.
- 27 Z. Yu, J. D. Schonhoft, S. Dhakal, R. Bajracharya, R. Hegde, S. Basu and H. Mao, *J. Am. Chem. Soc.*, 2009, **131**, 1876.
- 28 D. Koirala, S. Dhakal, B. Ashbridge, Y. Sannohe, R. Rodriguez, H. Sugiyama, S. Balasubramanian and H. Mao, *Nat. Chem.*, 2011, **3**, 782.
- 29 S. Dhakal, Y. Cui, D. Koirala, C. Ghimire, S. Kushwaha, Z. Yu, P. M. Yangyuoru and H. Mao, *Nucleic Acids Res.*, 2013, **41**, 3915.
- 30 M. de Messieres, J. C. Chang, B. Brawn-Cinani and A. La Porta, *Phys. Rev. Lett.*, 2012, **109**, 058101.
- 31 The change in the end-to-end distance across the transition is force-dependent. The force dependence may be neglected for small differences of force but based on the Worm-like chain model, they will need to be taken into account when extrapolating to zero-force.
- 32 J. D. Schonhoft, R. Bajracharya, S. Dhakal, Z. Yu, H. Mao and S. Basu, *Nucleic Acids Res.*, 2009, **37**, 3310.
- 33 M. Carrion-Vazquez, A. F. Oberhauser, S. B. Fowler, P. E. Marszalek, S. E. Broedel, J. Clarke and J. M. Fernandez, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 3694.