

Document downloaded from:

<http://hdl.handle.net/10251/152715>

This paper must be cited as:

Garavís, M.; Bocanegra, R.; Herrero-Galán, E.; González, C.; Villasante, A.; Arias-Gonzalez, JR. (2013). Mechanical unfolding of long human telomeric RNA (TERRA). *Chemical Communications*. 49(57):6397-6399. <https://doi.org/10.1039/c3cc42981d>



The final publication is available at
<https://doi.org/10.1039/c3cc42981d>

Copyright The Royal Society of Chemistry

Additional Information

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/chemcomm

COMMUNICATION

Mechanical unfolding of long human telomeric RNA (TERRA)

Miguel Garavís,^{‡ab} Rebeca Bocanegra,^{‡c} Elías Herrero-Galán,^c Carlos González,^{*,b} Alfredo Villasante,^{*,a} and J. Ricardo Arias-González^{*,de}

Received (in XXX, XXX) Xth XXXXXXXXXX 20XX, Accepted Xth XXXXXXXXXX 20XX

DOI: 10.1039/b000000x

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-quadruplexes blocks, which unfold at lower forces than their DNA counterparts.

Telomeres are nucleoprotein structures that protect chromosome ends from being recognized as DNA breaks.¹ Mammalian telomere DNA consists of long tandem arrays of double-stranded TTAGGG repeats that are maintained by the telomerase.² 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 presence in vivo 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.^{3,4} It has also been found that telomere end-binding proteins control the folding and unfolding of DNA G-quadruplexes in vitro⁵ and in vivo.⁴

Telomeres are transcribed from the subtelomeric regions towards chromosome ends into telomeric repeat-containing RNA (TERRA).^{6,7} These non-coding RNAs contain subtelomere-derived sequences and an average of 34 GGGUUA repeats at their 3' end.⁸ TERRA acts as scaffold for the assembly of telomeric proteins involved in telomere maintenance and telomeric heterochromatin formation.^{9,10} Importantly, there is also evidence for the presence of TERRA RNA G-quadruplexes in living cells.¹¹ It has been shown by nuclear magnetic resonance (NMR) spectroscopy^{12,13} and by X-ray crystallography¹⁴ that short TERRA molecules form parallel-stranded G-quadruplex structures but high-resolution structures of long telomeric RNAs have not yet been obtained. Electrospray mass spectrometry¹⁵ and computational analysis^{12,16} on long TERRA molecules have suggested stacking between their quadruplexes. The largest TERRA molecule (51nt) analyzed by NMR has showed intramolecular parallel G-quadruplex.¹⁶

Here, we report the synthesis and purification of TERRA molecules up to 96nt (twice de largest molecule previously analyzed by NMR), a size similar to the endogenous nuclear TERRA⁸ (see Supplementary Data). We have also used optical-tweezers (OT) to study RNA molecules with different number 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

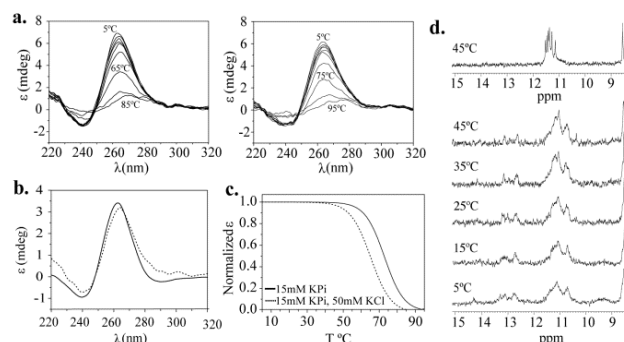


Fig. 1 a. Series of CD spectra of Q4 in 15 mM KPi pH 7 buffer (left) and 15 mM KPi pH 7, 50 mM KCl (right) at different temperature, [oligonucleotide] = 2 μ M. b. CD melting curves of Q4 at different K⁺ concentrations. c. CD spectra normalized for G-quadruplex concentration of r(UAGGGUUAGGGU) (straight 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 H₂O/D₂O 9:1 in 15 mM KPi pH 7 buffer. RNA concentration of 100 μ M and 10 μ M, respectively.

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.^{12,13,16-20} 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 RNAs in ammonium acetate buffer.¹⁵ On the other hand, the normalized CD spectra of the bimolecular G-quadruplex formed by r(UAGGGUUAGGGU) and Q4 showed similar shape and amplitude, which indicates that most of the Q4 molecules are forming four G-quadruplexes (Fig. 1b).²¹

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 buffer 25 mM potassium phosphate pH 7 and 25 mM potassium phosphate pH 7, 50 mM KCl respectively. Such notable difference between melting temperatures confirms the influence of potassium concentration on thermal stability of RNA G-quadruplexes.²² On the other hand, comparison of melting temperatures of Q4 and intramolecular telomeric RNA sequences reported in the bibliography^{17,18} suggests that the length of the

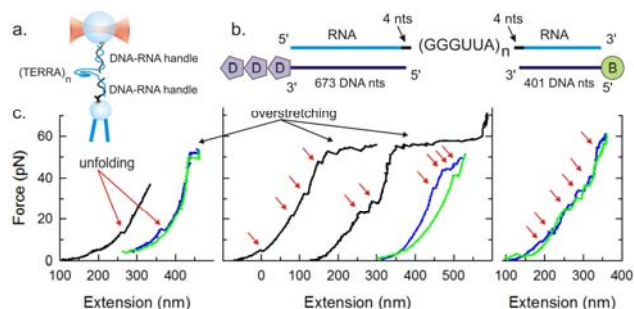


Fig. 2. **a.** Cartoon of the experiment: A Q_n construction is attached between a bead (biotin–streptavidin linkage), held by suction on the top of a micropipette, and an optically trapped bead (digoxigenin–anti-digoxigenin antibody linkage)²⁸. **b.** Cartoon of the OT construction showing each component 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.

sequence is not necessarily a factor that enhances thermal stability.

High scale synthesis and purification of long telomeric RNA were performed following previously described protocols²³ 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(UAGGGUAGGGU), 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 a 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 RNA¹⁴ or DNA quadruplex.²⁴

The vectors employed to produce telomeric RNA in sufficiently large amount for NMR experiments were also used to generate the constructs required for OT analysis (Fig. 2a and Supplementary data). 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,^{25,26} 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 to what was described for DNA quadruplexes.^{27–29} Typical rupture forces are 15 ± 2 pN, below those observed for DNA quadruplexes for the experimental loading rate used here (22 ± 2 pN/s, see Supplementary Data).^{27,30} Weaker stacking interactions between adjacent G-tetrad planes in

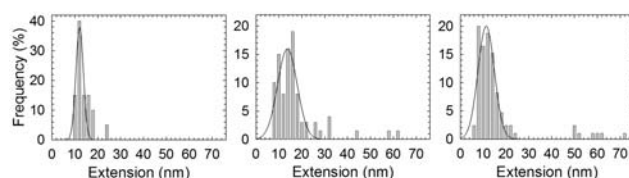


Fig. 3 Unfolding extension histograms for Q1 (left, $n=20$), Q4 (center, $n=68$) and Q6 (right, $n=85$) TERRA repeats from single-molecule experiments. Peak position (and standard deviation) according to Gaussian fits to the experimental histograms are 12.1 (1.6) nm, 13.7 (4.2) nm and 11.3 (3.6) nm,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

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. 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.²⁵ 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.²⁷ 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.²⁷

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.³¹ 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, what typically resulted in molecular breakages of the single-molecule constructs at high forces. However, some of the constructions could resist forces beyond the overstretching transition of the hybrid handles. This transition provides here a control check to show that single-molecule constructs were used in the OT experiments. An analysis of this transition shows that hybrid DNA:RNA molecules approximately overstretch as an A-form molecule.²⁵

The fact that 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 ~12 nm, with no apparent mixture of populations.^{27,31} 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 Supplementary Data), 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 for DNA quadruplexes,³² 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 preparations³³ than other cooperative transitions in DNA or RNA (overstretching or unzipping).²⁶

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 mechanical 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 form higher order structures at a single molecule level, which exhibit some cooperative unfolding events.

This work was supported by grants from the Spanish Ministry of Science and Innovation (grants RYC2007-01765 to JRA-G BFU2011-30295-C02-01 to AV, and CTQ2010-21567-C02-02 to CG). MG was supported by the FPI fellowship BES-2009-027909. RB and EH-G were supported by Comunidad de Madrid, grant CAM-S2009MAT-1507. AV acknowledges an institutional grant from the Fundación Ramón Areces to the CBMSO. JRAG wants to thank to Prof. J.L. Carrascosa and Prof. J.M. Valpuesta (CNB-CSIC) for their continuous support and encouragement in this research. We also acknowledge the excellent technical assistance of Beatriz de Pablos (CBMSO).

Notes and references

^aInstituto de Química Física Rocasolano, CSIC, C/Serrano 119, 28006 Madrid. Fax: (+34)915642431 Tel: (+34)915619400 E-mail: cgonzalez@iqfr.csic.es

^bCentro de Biología Molecular “Severo Ochoa” CSIC-UAM, Universidad Autónoma de Madrid, Nicolás Cabrera 1, 28049 Madrid, Spain. Fax: (+34)911964420 Tel: (+34)911964401 E-Mail: avillasante@cbm.uam.es

^cCentro Nacional de Biotecnología (CNB-CSIC), C/ Darwin 3, Cantoblanco. 28049 Madrid, Spain.

^dIMDEA Nanociencia, C/ Faraday 9, Cantoblanco. 28049 Madrid. Fax: (+34)912998725 Tel: (+34)912998860 E-mail:ricardo.arias@imdea.org

^eCNB-CSIC-IMDEA Nanociencia Associated Unit “Unidad de Nanobiotecnología

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

‡ M. Garavis and R. Bocanegra made equal contribution to this paper.

- de Lange, T. *Genes Dev* 2005, 19, 2100.
- Blackburn, E. H. *Nature* 1991, 350, 569.
- Biffi, G.; Tannahill, D.; McCafferty, J.; Balasubramanian, S. *Nat Chem* 2013, 5, 182.
- Paeschke, K.; Simonsson, T.; Postberg, J.; Rhodes, D.; Lipps, H. J. *Nat Struct Mol Biol* 2005, 12, 847.
- Hwang, H.; Buncher, N.; Opresko, P. L.; Myong, S. *Structure* 2012, 20, 1872.
- Azzalin, C. M.; Reichenbach, P.; Khoraiuli, L.; Giulotto, E.; Lingner, J. *Science* 2007, 318, 798.
- Schoeftner, S.; Blasco, M. A. *Nat Cell Biol* 2008, 10, 228.
- Porro, A.; Feuerhahn, S.; Reichenbach, P.; Lingner, J. *Mol Cell Biol* 2010, 30, 4808.
- Deng, S.; Norseen, J.; Wiedmer, A.; Riethman, H.; Lieberman, P. M. *Mol Cell* 2009, 35, 403.
- Lopez de Silanes, I.; Stagno d'Alcontres, M.; Blasco, M. A. *Nat Commun* 2010, 1, 33.
- Xu, Y.; Suzuki, Y.; Ito, K.; Komiyama, M. *Proc Natl Acad Sci U S A* 2010, 107, 14579.
- Martadinata, H.; Phan, A. T. *J Am Chem Soc* 2009, 131, 2570.
- Xu, Y.; Kaminaga, K.; Komiyama, M. *J Am Chem Soc* 2008, 130, 11179.
- Collie, G. W.; Haider, S. M.; Neidle, S.; Parkinson, G. N. *Nucleic Acids Res* 2010, 38, 5569.
- Collie, G. W.; Parkinson, G. N.; Neidle, S.; Rosu, F.; De Pauw, E.; Gabelica, V. *J Am Chem Soc* 2010, 132, 9328.
- Martadinata, H.; Heddi, B.; Lim, K. W.; Phan, A. T. *Biochemistry* 2011, 50, 6455.
- Arora, A.; Maiti, S. *J Phys Chem B* 2009, 113, 10515.
- Joachimi, A.; Benz, A.; Hartig, J. S. *Bioorg Med Chem* 2009, 17, 6811.
- Qi, J.; Shafer, R. H. *Biochemistry* 2007, 46, 7599.
- Randall, A.; Griffith, J. D. *J Biol Chem* 2009, 284, 13980.
- Petraccone, L. In *Quadruplex Nucleic Acids*; Chaires, J. B., Graves, D., Eds.; Springer Berlin Heidelberg: 2013; Vol. 330, p 23.
- Kumari, S.; Bugaut, A.; Balasubramanian, S. *Biochemistry* 2008, 47, 12664.
- McKenna, S. A.; Kim, I.; Puglisi, E. V.; Lindhout, D. A.; Aitken, C. E.; Marshall, R. A.; Puglisi, J. D. *Nat Protoc* 2007, 2, 3270.
- Parkinson, G. N.; Lee, M. P.; Neidle, S. *Nature* 2002, 417, 876.
- Herrero-Galan, E.; Fuentes-Perez, M. E.; Carrasco, C.; Valpuesta, J. M.; Carrascosa, J. L.; Moreno-Herrero, F.; Arias-Gonzalez, J. R. *J Am Chem Soc* 2013, 135, 122.
- Bustamante, C.; Bryant, Z.; Smith, S. B. *Nature* 2003, 421, 423.
- Yu, Z.; Schonhoft, J. D.; Dhakal, S.; Bajracharya, R.; Hegde, R.; Basu, S.; Mao, H. *J Am Chem Soc* 2009, 131, 1876.
- Koirala, D.; Dhakal, S.; Ashbridge, B.; Sannohe, Y.; Rodriguez, R.; Sugiyama, H.; Balasubramanian, S.; Mao, H. *Nat Chem* 2011, 3, 782.
- Dhakal, S.; Cui, Y.; Koirala, D.; Ghimire, C.; Kushwaha, S.; Yu, Z.; Yangyuru, P. M.; Mao, H. *Nucleic Acids Res* 2013.
- de Messieres, M.; Chang, J. C.; Brawn-Cinani, B.; La Porta, A. *Phys Rev Lett* 2012, 109, 058101.
- 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.
- Schonhoft, J. D.; Bajracharya, R.; Dhakal, S.; Yu, Z.; Mao, H.; Basu, S. *Nucleic Acids Res* 2009, 37, 3310.
- Carrion-Vazquez, M.; Oberhauser, A. F.; Fowler, S. B.; Marszalek, P. E.; Broedel, S. E.; Clarke, J.; Fernandez, J. M. *Proc Natl Acad Sci U S A* 1999, 96, 3694.