Bioorganic & Medicinal Chemistry Letters 20 (2010) 2350–2353

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A reverse transcriptase stop assay revealed diverse quadruplex formations in UTRs in mRNA

Masaki Hagihara [a], Keisuke Yoneda [a], Hiroaki Yabuuchi [b], Yasushi Okuno [b], Kazuhiko Nakatani [a,*]

[a] Department of Regulatory Bioorganic Chemistry, The Institute of Scientific and Industrial Research (ISIR), Osaka University, Ibaraki 567-0047, Japan
[b] Department of Systems Bioscience for Drug Discovery, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan

ARTICLE INFO

Article history:
Received 30 November 2009
Revised 21 January 2010
Accepted 25 January 2010
Available online 17 February 2010

Keywords:
RNA quadruplex
Reverse transcriptase
mRNA

ABSTRACT

Here, we developed a reverse transcriptase based method (*RTase* stop assay) to characterize quadruplex formations in guanine-rich RNAs with high sensitivity and specificity. By using the *RTase* stop assay, we also revealed a plausible structural polymorphism in biologically important RNAs. The *RTase* stop assay would provide helpful insight into RNA quadruplex structures and functions, together with other analytical methods, including various footprinting techniques.

A wide variety of RNA plays an important role in the regulation of biological function within cells. The function of RNA largely depends on tertiary structures, in which RNA molecules use specific hydrogen bonds, including Watson–Crick and Hoogsteen bonds, to form active structures. Among a variety of RNA ternary folded structures, quadruplexes are one of the unique structures, in which guanine tetrads make Hoogsteen hydrogen bonds with each other in plane with incorporating metal ion, such as potassium and sodium, inside the tetrads.[1–3] Recent reports have demonstrated that quadruplex structures in untranslated regions (UTRs) in mRNAs are involved in the translational regulation in vitro and vivo.[4–6]

Computational predictions[7] together with various analytical methods, including spectroscopic methods[8,9] and footprinting techniques,[10–12] are commonly used to deduce secondary and tertiary structures of RNAs. Each technique has inherent advantages and disadvantages with regard to the quadruplex formations in RNAs, although spectroscopic analyses by using model oligonucleotides suggested that guanine-rich RNAs are easy to form quadruplex structures.[4–6,13] Simple and sensitive methods addressing RNA quadruplexes would provide deeper understandings of RNA quadruplex structures and functions.

Reverse transcriptases have been used to predict stable secondary structures in RNAs, involving quadruplex structures, however, the sensitivities of these assays are sometimes too low to fully address the sequence–structure relationships of quadruplex formation.[14,15] Here, we reported a reverse transcription based method to precisely evaluate the stability and the structural diversity of quadruplexes in RNAs.

In order to gain insight into local quadruplex structures on an RNA template, we studied an RNA-dependent DNA polymerase stop assay, named a reverse transcriptase stop assay (*RTase* stop assay) (Fig. 1A). In the *RTase* stop assay, reverse transcriptases, such as those originated from Moloney murine leukemia virus (M-MuLV), proceeded along RNA templates until the enzyme encountered stable RNA quadruplex structures. Interference of *RTase* reaction by quadruplex structures on the templates would result in the production of truncated complement DNA products, which can be detected by PAGE analyses.[16,17]

First, a human telomeric sequence 5′-r(UUAGGG)$_4$UUA-3′ (**R-telo27**) was chosen as a model of RNA quadruplexes.[13,18] **R-telo27** showed UV-melting curves with an inverse transition at 295 nm.[19] The melting temperature showed a significant KCl concentration-dependency (Fig. 1B) with T_m values of 61.7 ± 0.2 °C and 76.5 ± 0.3 °C in 10 and 100 mM KCl, respectively. CD spectra of **R-telo27** in the both KCl solutions suggested a parallel stranded quadruplex conformer, exhibiting a strong positive peak at 263 nm and a relatively weak peak near 240 nm (Fig. 1C). These data suggested that **R-telo27** having a four-telomeric-repeat afforded quadruplex structures with a different stability, depending on the KCl concentrations.

With the biophysical information of quadruplex stabilities in hand, we next investigated quadruplex formations by the *RTase* stop assay on RNA templates that contained the **R-telo27** sequence at the center of the template. In 100 mM KCl, the *RTase* reaction was exclusively interrupted at the first GGG site from 3′-end of the templates, where a plausible quadruplex was formed, regard-

* Corresponding author.
E-mail address: nakatani@sanken.osaka-u.ac.jp (K. Nakatani).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.01.158

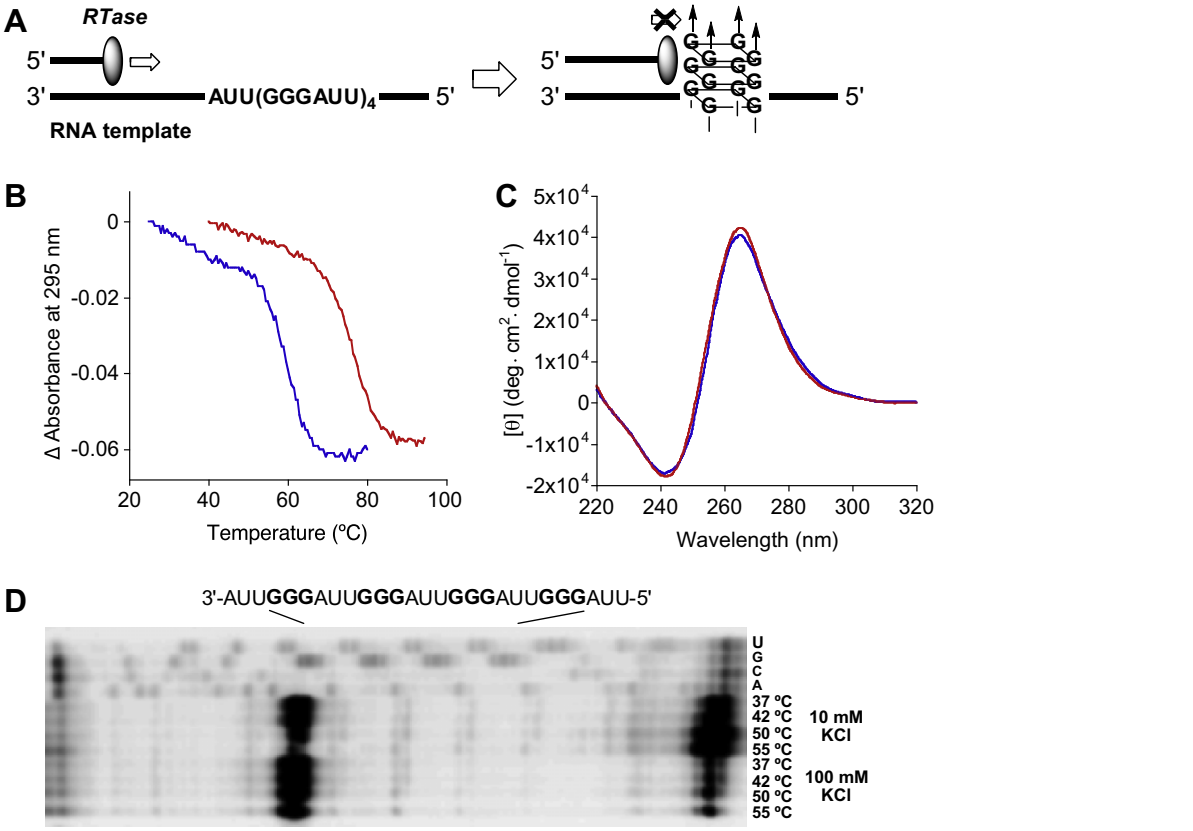

Figure 1. (A) Schematic illustration of a reverse transcriptase stop assay on a four-telomeric-repeat containing RNA. (B, C) Biophysical properties of a four-telomeric-repeat RNA (**R-telo27**). (B) T_m melting curves of **R-telo27** and (C) CD spectra of **R-telo27**. All experiments were carried out at 5 μM **R-telo27** in the 10 mM sodium cacodylate buffer (pH 7.0) containing 10 mM (blue) and 100 mM (red) KCl at 25 °C. (D) Interruption of *RTase*-mediated DNA synthesis with different reaction temperatures on RNA templates in the presence of KCl. The lane markers U, G, C, and A indicate the bases on the template strand. Guanines in the telomeric-repeat regions were emphasized in bold.

less of increasing temperatures from 37 to 55 °C (Fig. 1D). While, in 10 mM KCl, the paused products gradually decreased concomitant with increasing reaction temperature. These observations are well correlated with the decreased stability of quadruplex structure observed in the melting analysis. In contrast, no apparent paused bands were detected for *RTase* reaction on the RNA template containing a hairpin-loop structure (see Fig. S1 in Supplementary data), suggesting that the quadruplex structures serve as an effective inhibitor of *RTase* possibly due to their aberrant structures.

To certify the quadruplex formations in the four-telomeric-repeat RNA, a series of RNA variants (Table 1) was tested by the *RTase* stop assay. These variants had a deletion of a GGG sequence (*D*IV), single guanine mutations to adenine in each GGG sequence (mut I, II, III, and IV), or an insertion of the GGG upstream of the guanine-rich region of *D*IV (mut V) with an entire RNA length kept unchanged.

As shown in Figure 2, (1) the *RTase* reaction was not at all interfered on the RNA template containing a three-telomeric-repeat (*D*IV), (2) the variants containing even the single guanine-to-adenine mutations inside each GGG sequence did not show any paused products (mut I, II, III, and IV), and (3) the paused bands were clearly observed by the insertion of the GGG at the nine-base upstream of the position IV. The remarkable differences with regard to the number of repeats would attribute to capabilities of the quadruplex formations in the RNA templates. Considering that stable quadruplex formations generally require an association of four strands having at least three-guanine-repeat, the *RTase* stop assay would correctly provide information of an intrastranded RNA quadruplex formation. Also, these results suggested that RNA quadruplex structures were tolerant to the linker-length between GGG units, possibly due to a flexible feature of single-stranded RNAs. Taken together, the *RTase* stop assay revealed an intrinsic propensity for RNA quadruplex formations.

Next, we investigated the formation of quadruplex in UTRs by the *RTase* stop assay. Bioinformatic analysis suggested genetically conserved potential quadruplex structure sequences (PQSSs) both

Table 1
Telomeric RNA mutants used in this study

3′-	I		II		III		IV		V		-5′
AUU	**GGG**	AUU	**GGG**	AUU	**GGG**	AUU	**GGG**	AUU			Wild-type
AUU	**GGG**	AUU	**GGG**	AUU	**GGG**	AUU	**AAA**	AUU			ΔIV
AUU	**GGG**	AUU	**GGG**	AUU	**GGG**	AUU	**GAG**	AUU			mut IV
AUU	**GGG**	AUU	**GGG**	AUU	**GAG**	AUU	**GGG**	AUU			mut III
AUU	**GGG**	AUU	**GAG**	AUU	**GGG**	AUU	**GGG**	AUU			mut II
AUU	**GAG**	AUU	**GGG**	AUU	**GGG**	AUU	**GGG**	AUU			mut I
AUU	**GGG**	AUU	**GGG**	AUU	**GGG**	AUU	**AAA**	AUU	UCGCAU	**GGG**	mut V

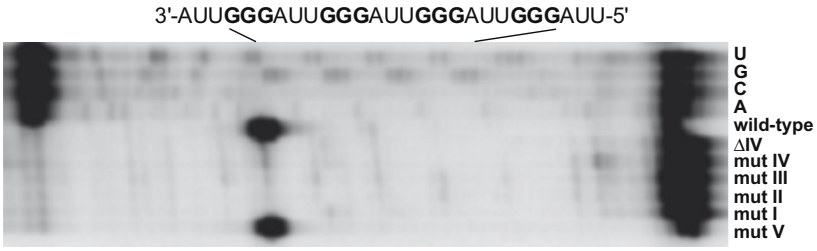

Figure 2. Quadruplex formations in the various telomeric-repeat mutants. The *RTase* reaction was performed at 42 °C in 100 mM KCl buffer. The lane markers U, G, C, and A indicate the bases on the template strand of the wild-type RNA. Guanines in the telomeric-repeat regions were emphasized in a bold case.

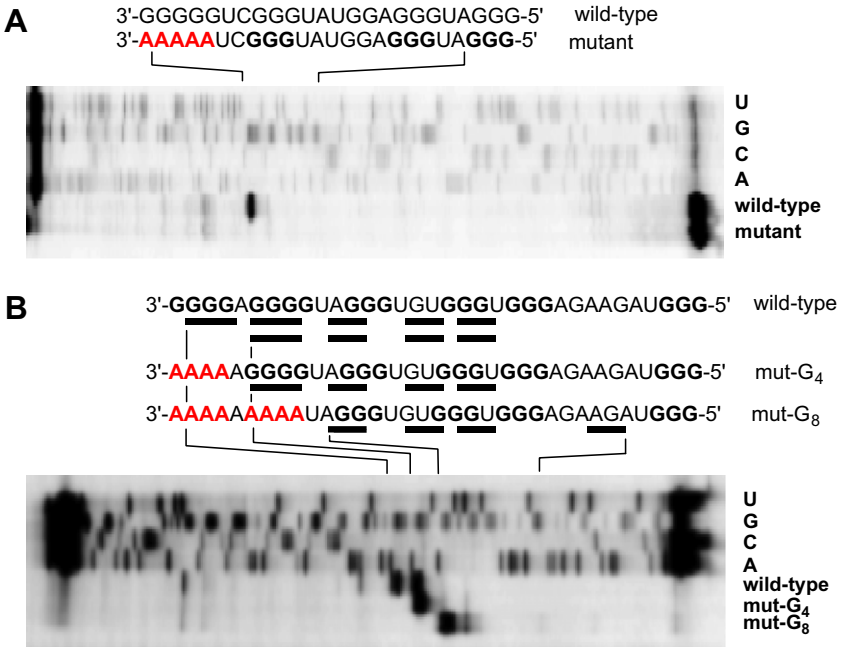

Figure 3. Quadruplex formations in the PQSS in 5′- and 3′-UTRs. Probing quadruplex formation (A) in the wild-type *GLI* 3′-UTR and its mutant and (B) in the *INHA* 5′-UTR. Bar indicated the PQSSs in the UTRs. The lane markers U, G, C, and A indicate the bases on the template strand of the wild-type UTRs.

in 5′- and 3′-UTRs. We focused on the two PQSSs in the human transcription factor Gli1 (*GLI*) 3′-UTR and in the inhibin-A (*INHA*) 5′-UTR. The *GLI* encoded a zinc finger protein that mediates Hedgehog signaling at the end of the pathway, and activation of Gli1 protein has been shown to be responsible for tumorigenesis.[20] The *INHA* encodes peptide hormone that inhibits the production of follicle-stimulating hormone (FSH) by the pituitary gland. The inhibins play roles in the control of gametogenesis, and embryonic and fetal development.[21]

The *GLI* 3′-UTR exclusively gave paused bands at the 3′-side of the PQSS (5′-**GGG**AU**GGG**AGGUAU**GGG**CU**GGGGG**-3′) (Fig. 3A). A GGGGG-to-AAAAA mutation (5′-**GGG**AU**GGG**AGGUAU**GGG**CU**AAAAA**-3′) in the PQSS completely disrupted the formation of quadruplex as observed in the wild-type *GLI* 3′-UTR. This observation suggested the stable quadruplex formation in the guanine-rich *GLI* PQSS.

While, the *INHA* 5′-UTR gave two major paused bands in the PQSS (5′-**GGG**UAGAAGA**GGG**U**GGG**UGU**GGG**AU**GGGGA**GGGG-3′) (Fig. 3B, lane 5). Sequential mutations of guanine-repeat units from the 3′-side of the PQSS moved the formation of paused products to 5′-side of the templates (Fig. 3B, lanes 6 and 7). Furthermore, all the paused products were assigned to be at the 3′-side of the guanine-repeat in the PQSS, implying that guanine-repeat regions can afford a variety of quadruplex formations in UTRs.

The *RTase* stop assay described here provided direct evidence for quadruplex formation in these UTRs, as well as information regarding the structural diversity of quadruplexes in the PQSS. The resolution of the *RTase* stop assay is high enough to address the structural diversity of quadruplexes, which would be feasible in many other guanine-rich regions in UTRs.

Acknowledgement

This work was supported by the Human Frontier Science Program Award HFSP RGY79/2007

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.158.

References and notes

1. Burge, S.; Parkinson, G. N.; Hazel, P.; Todd, A. K.; Neidle, S. *Nucleic Acids Res.* **2006**, *34*, 5402.
2. Patel, D. J.; Phan, A. T.; Kuryavyi, V. *Nucleic Acids Res.* **2007**, *35*, 7429.
3. Lane, A. N.; Chaires, J. B.; Gray, R. D.; Trent, J. O. *Nucleic Acids Res.* **2008**, *36*, 5482.

4. Kumari, S.; Bugaut, A.; Huppert, J. L.; Balasubramanian, S. *Nat. Chem. Biol.* **2007**, *3*, 218.
5. Arora, A.; Dutkiewicz, M.; Scaria, V.; Hariharan, M.; Maiti, S.; Kurreck, J. *RNA* **2008**, *14*, 1.
6. Wieland, M.; Hartig, J. S. *Chem. Biol.* **2007**, *14*, 757.
7. Zuker, M. *Nucleic Acids Res.* **2003**, *31*, 3406.
8. Scott, L. G.; Hennig, M. *Methods Mol. Biol.* **2008**, *452*, 29.
9. Golden, B. L. *Methods Mol. Biol.* **2007**, *363*, 239.
10. Ryder, S. P.; Ortoleva-Donnelly, L.; Kosek, A. B.; Strobel, S. A. *Methods Enzymol.* **2000**, *317*, 92.
11. Tullius, T. D.; Greenbaum, J. A. *Curr. Opin. Chem. Biol.* **2005**, *9*, 127.
12. Soukup, G. A.; Breaker, R. R. *RNA* **1999**, *5*, 1308.
13. Sacca, B.; Lacroix, L.; Mergny, J. L. *Nucleic Acids Res.* **2005**, *33*, 1182.
14. Nielsen, F. C.; Christiansen, J. *J. Biol. Chem.* **1992**, *267*, 19404.
15. Schaeffer, C.; Bardoni, B.; Mandel, J. L.; Ehresmann, B.; Ehresmann, C.; Moine, H. *EMBO J.* **2001**, *20*, 4803.
16. Han, H.; Hurley, L. H.; Salazar, M. *Nucleic Acids Res.* **1999**, *27*, 537.
17. Hagihara, M.; Goto, Y.; Nakatani, K. *ChemBioChem* **2008**, *9*, 510.
18. Xu, Y.; Kaminaga, K.; Komiyama, M. *J. Am. Chem. Soc.* **2008**, *130*, 11179.
19. Mergny, J. L.; Phan, A. T.; Lacroix, L. *FEBS Lett.* **1998**, *435*, 74.
20. Stecca, B.; Ruiz, I.; Altaba, A. *J. Neurobiol.* **2005**, *64*, 476.
21. Kitisin, K.; Saha, T.; Blake, T.; Golestaneh, N.; Deng, M.; Kim, C.; Tang, Y.; Shetty, K.; Mishra, B.; Mishra, L. *Sci. STKE.* **2007**, *39*, cm1.