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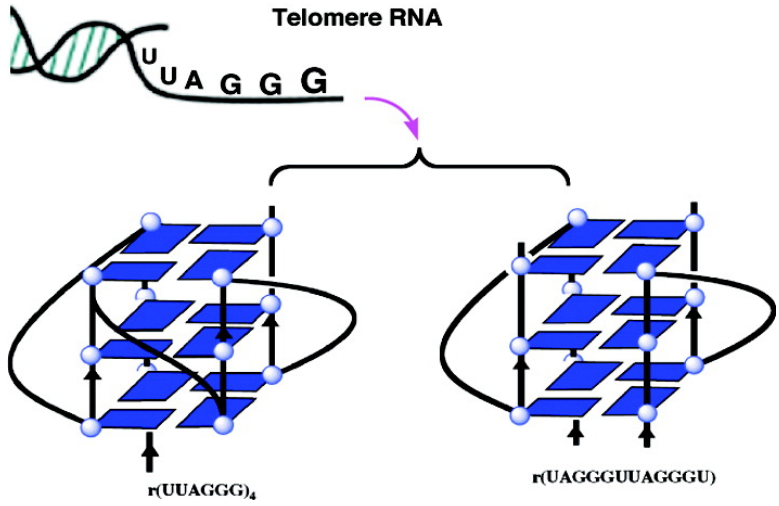
J. Am. Chem. Soc., **2008**, 130 (33), 11179-11184 • DOI: 10.1021/ja8031532 • Publication Date (Web): 19 July 2008



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G-Quadruplex Formation by Human Telomeric Repeats-Containing RNA in Na^+ Solution

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Abstract: For a long time, telomeres have been considered to be transcriptionally silent. Very recently, a breaking finding from two groups demonstrated that telomere DNA is transcribed into telomeric repeat-containing RNA in mammalian cells (Azzalin, C. M.; Reichenbach, P.; Khoriauli, L.; Giulotto, E.; Lingner, J. *Science* **2007**, *318*, 798−801. Schoefter, S.; Blasco, M. A. *Nat. Cell Biol.* **2008**, *10*, 228−236). The telomeric RNA, a newly appeared player in telomere biology, may be a key component of telomere machinery. In the current study, we used a combination of NMR, circular dichroism (CD), matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOFMS), and gel electrophoresis to investigate the structural features of a human telomere RNA sequence. We demonstrated that human telomere RNA can form a parallel G-quadruplex structure in the presence of Na^+. Importantly, we found for the first time that the G-quadruplex forming telomere RNA protects itself from enzymatic digestion. These results provide valuable information to allow understanding of the structure and function of human telomeric RNA.

Introduction

Telomeres are essential structures at the ends of all eukaryotic chromosomes. In human, telomeric DNA consists of a duplex region composed of TTAGGG repeats, ending in a shorter G-rich single-stranded overhang.[1–6] Telomeres play an important role in genome stability and cell growth by protecting chromosome ends.[1,7,8] Tumor formation and aging have been linked to alterations at the telomere.[9–12] Telomere DNA are also the substrate for telomerase, which elongates chromosome ends by adding G-rich repeats.[1,13] Recent studies have suggested that the human telomere DNA may exist in multiple states such as the G-quadruplex or T-loop.[14–21] For example, we and other two groups have demonstrated that a 22 nt human telomeric sequence formed a unique topology of the G-quadruplex.[17–20] Such quadruplex structures have been suggested to represent a potential tumor-selective target for chemotherapy.[22–29]

Very recently, two studies demonstrated that telomere DNA is transcribed into telomeric repeat-containing RNA in mammalian cells.[30,31] Telomeric RNAs were detected in different human and rodent cell lines, containing mainly UUAGGG repeats in the heterogeneous length. It has been suggested that telomere RNAs are transcribed from several subtelomeric loci toward chromosome ends and are localized to the telomere DNA. These findings raise a crucial question of how telomeric RNA is specifically associated with chromosome ends. The existence of telomeric RNAs may provide a new level of regulation and protection of chromosome ends that could facilitate new insight into fundamental biological processes such

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as cancer and aging. Revealing the structure and function of telomere RNA will be essential for understanding telomere biology and telomere-related diseases, such as telomere loss and premature aging. In several of previous studies it has been reported that associated cations can determine the type of G-quadruplex formed.[32,33] For example, a 22 nt human telomere DNA sequence folds into an antiparallel-type G-quadruplex structure in the presence of Na^+ ions,[14] whereas the same 22 nt sequence adopts a different mixed-type structure in the presence of K^+ ions.[17–20] However, the effect of such metal cations on the conformation of human telomeric RNA has not yet been elucidated. In the present study, we have investigated the structural features of the human telomere RNA sequence. We found that human telomere RNA can form a parallel G-quadruplex structure in the presence of Na^+ and that formation of the G-quadruplex resists telomere RNA degradation. Multimethod approaches, CD, NMR, MALDI-TOFMS, and gel electrophoresis, have been used to provide several complementary lines of evidence for telomere RNA G-quadruplex formation. The possible implications for the structure in chromosome ends are discussed.

Results and Discussion

Circular Dichroism (CD) Studies on Human Telomere RNA Sequence. The typical bands in the CD spectra show fundamental characteristics allowing G-quadruplex structures to be distinguished.[34–41] To define the structural features of human telomere RNA sequence, we examined the conformation of the 12 nt human telomere RNA sequence r(UAGGGUUAGGGU) ORN-1 by CD spectroscopy. The CD spectrum of ORN-1 in the presence of Na^+ at 25 °C showed a positive band at 265 nm and a negative band at 240 nm (Figure 1a) which are the characteristic CD signature of a parallel G-quadruplex structure of RNA.[34,42,43] We noted that the dominant CD band at 265 nm was extinguished at 90 °C consistent with an unstructured single strand, indicating that the telomere RNA G-quadruplex is formed at 25 °C (Figure 1a). To further understand the molecular basis of the telomere RNA G-quadruplex structure, we investigated the structure of the human 24 nt telomere RNA sequence $r(UUAGGG)_4$ (ORN-2). As shown in Figure 1b, the 24 nt RNA exhibits a CD spectrum characteristic of the parallel G-quadruplex also, while a dominant CD band at 265 nm was extinguished at 90 °C. CD spectra of ORN-1 and ORN-2 at the base concentrations 0.02, 0.05, 0.1, and 0.2 mM in Na^+ were measured, respectively. There is an increase in the 265 nm band with an increase in RNA concentration, indicating the increased population of parallel structures (Supporting Information, Figure S1). Ac-

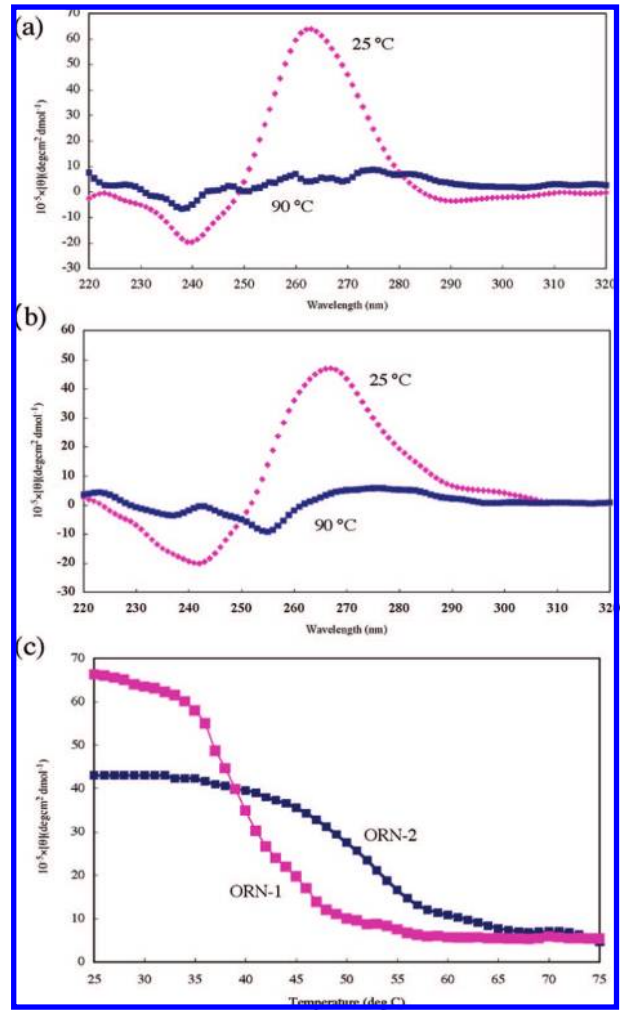

Figure 1. (a) CD spectra of r(UAGGGUUAGGGU) ORN-1 in the presence of 100 mM NaCl at 25 and 90 °C. (b) CD spectra of $r(UUAGGG)_4$ ORN-2 in the presence of 100 mM NaCl at 25 and 90 °C. (c) CD melting curves for ORN-1 and ORN-2 monitored at 265 nm in the presence of 100 mM NaCl.

cording to CD melting experiments, the T_m value of the parallel G-quadruplexes in 100 mM NaCl solution was 41 ± 1 °C and 51 ± 1 °C, suggesting that ORN-1 or ORN-2 forms a stabile G-quadurplex under low-salt conditions (Figure 1c). The melting profile of ORN-2 was reversible, whereas the hysteresis was observed in ORN-1 leading to nonsuperimposable heating and cooling curves (Figure S2). We also examined the structural features of the two-repeats human telomere RNA sequence r(UUAGGGUUAGGG) ORN-3 that has two small changes in the 3′ and 5′ ends of the sequence compared to ORN-1. ORN-3 gives a positive CD band at 265 nm and a negative band at 240 nm in the presence of Na^+, indicating that the parallel G-quadruplex structure is formed (Figure S3). The result suggested that the single base mutations in the 3′ and 5′ ends of the G-quadruplex did not influence the conformation of the telomere RNA G-quadruplex structure.

NMR Studies on 12 nt Human Telomere RNA Sequence. G-Quadruplex structures display characteristic resonances for the imino protons,[44–46] whose presence may be used to confirm the presence of the G-quadruplex structure in solution. We investigated the structure of human 12 nt telomere RNA ORN-1 by NMR. In the imino proton region of the 600 MHz ^1H NMR

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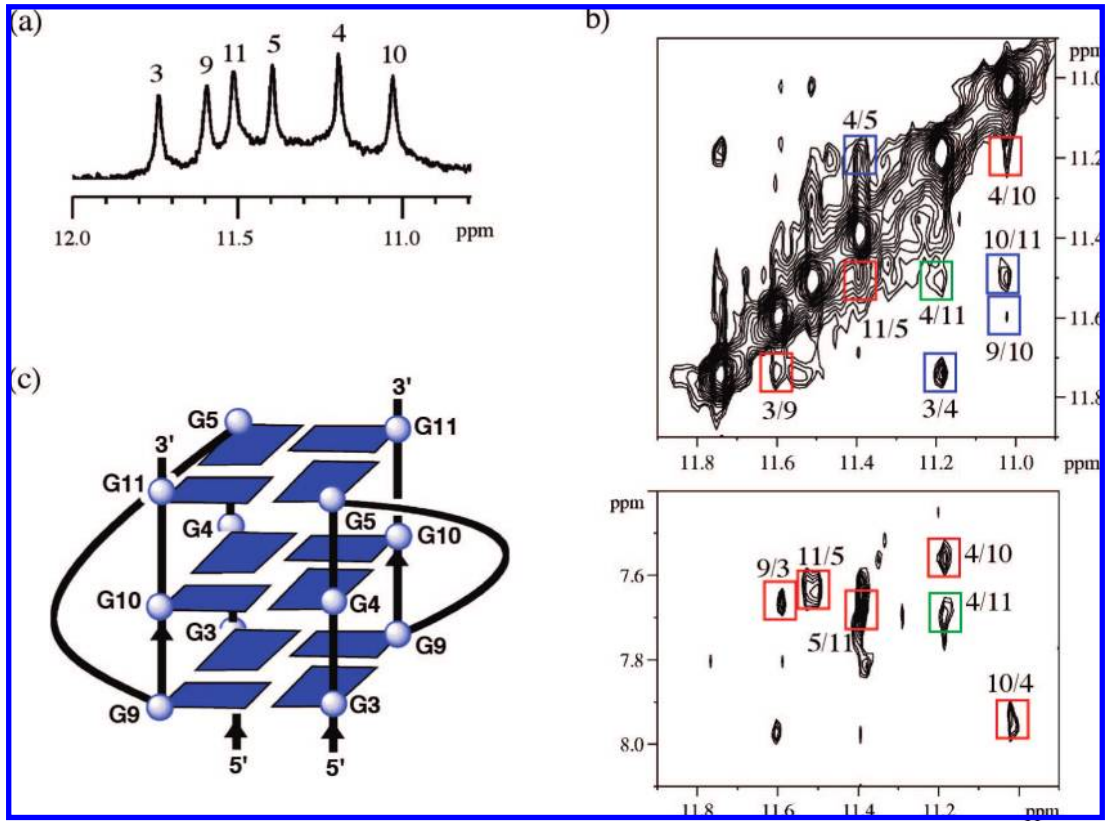

Figure 2. (a) One-dimensional 600 MHz imino proton spectrum of the telomere RNA ORN-1 in 90% H_2O/10% D_2O solution containing 200 mM NaCl and 10 mM Na-phosphate, pH 6.8, 25 °C. (b) H1−H1 region (top) and H1−H8 region (bottom) of 2D-NOESY spectrum of ORN-1 in the presence of 200 mM NaCl and 10 mM Na-phosphate; intratetrad, sequential connectivity, and intertetrad were represented by red, blue, and green boxes, respectively. (c) Schematic structure of human telomeric repeat-containing RNA (ORN-1) 5′-$U_1A_2G_3G_4G_5U_6U_7A_8G_9G_{10}G_{11}U_{12}$-3′, where blue boxes represent guanine bases in the anti conformation.

spectrum of ORN-1 in the presence of Na^+, six sharp peaks assignable to the guanine imino protons of the G-quadruplex structure were observed at 11.0−12.0 ppm (Figure 2a). These six signals corresponding to 12 G residues indicate that ORN-1 forms a symmetric dimer in a Na^+ solution. Furthermore, the folding topology of a dimeric G-quadruplex was determined based on the NOE connectivities of H1−H1 and H1−H8. For example, the intratetrad NOE connections G11H1/G5H1 (GH1/GH1) and G5H1/G11H8, G11H1/G5H8 (GH1/GH8) revealed the formation of G-tetrad G5-G11-G5-G11 (Figure. 2b). Using the same method, two other G-tetrads, G4-G10-G4-G10 and G3-G9-G3-G9, were also defined (Figure 2b). The sequential NOE interactions G9H1/G10H1, G10H1/G11H1, G3H1/G4H1, and G4H1/G5H1 correspond to imino protons of G on adjacent G-tetrads for G9-G10-G11 and G3-G4-G5 steps on each of the four strands (Figure 2b), indicating the same G-arrangement and glycosidic conformations (anti−anti steps) of three G-tetrad planes. No strong H8/H1′ NOE cross peaks were observed for all G's, indicating that the G's were the anti conformation (Figure S4). The intertetrad NOE connections were used to further define the G-quadruplex alignment. For example, the NOE interactions of G4H1/G11H1 and G4H1/G11H8 connect the top and middle G-tetrad planes (Figure 2b). It is concluded that the telomere RNA ORN-1 forms a parallel dimeric G-quadruplex with double-chain-reversal loops in the presence of Na^+ ions (Figure 2c), similar to the crystal and NMR structure of human telomeric DNA.[15,44] NMR methods have long been used to study quadruplexes in DNA.[14,45,47–52] There are only a few reports, however, on NMR studies of an RNA G-quadruplex in solution.[53,54] We note that the 12 nt telomere RNA sequence exhibits a 1D NMR spectrum with well-defined peaks in the imino proton region. These 6 protons correspond exactly to the 12 imino protons involved in interguanine hydrogen bonding within the G-quartet and are unequivocal evidence for three G-quartet planes in the symmetric dimer structure.

MALDI-TOFMS Studies on 12 nt Human Telomere RNA Sequence. Recent advances in mass spectrometry (MS) have led to a successful technique for the investigation of noncovalent

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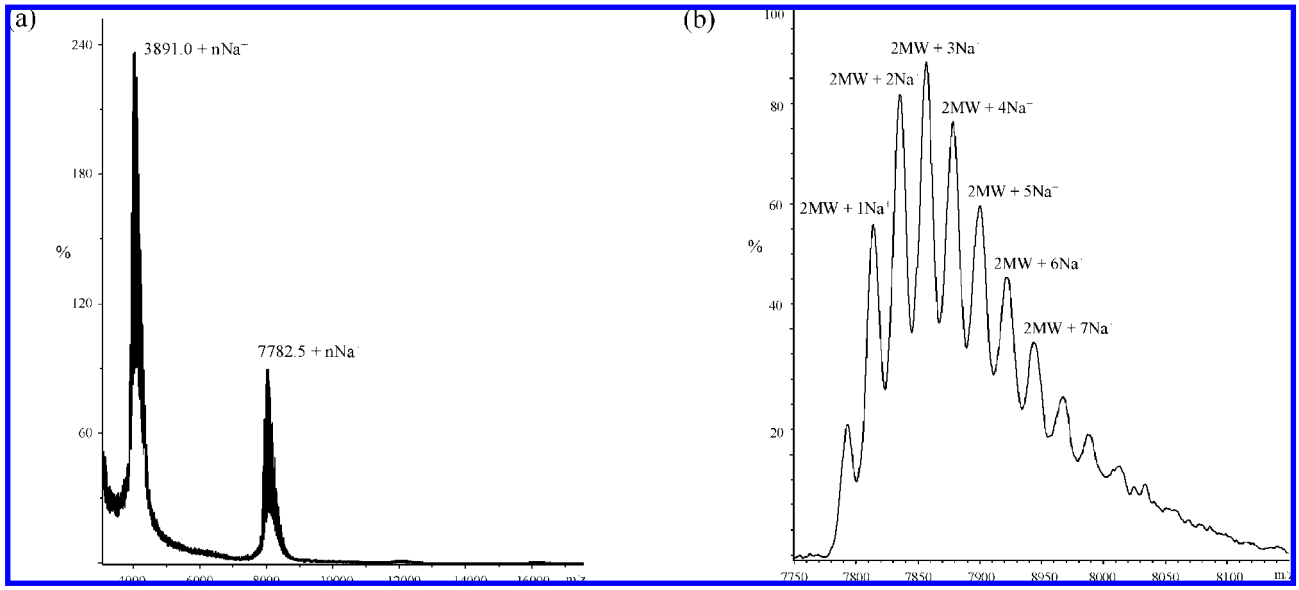

Figure 3. (a) Low-resolution MALDI-TOFMS spectrum of ORN-1 in the presence of 200 mM NaCl. (b) Expansion of high-resolution region of dimeric ORN-1; the numbers of Na^+ ion adducts are shown.

intermolecular complexes of DNA, PNA, and PNA−DNA hybrids.[55–58] To further characterize the RNA G-quadruplex structure, MALDI-TOFMS was used to directly observe the G-quadruplex formation. Figure 3 shows the spectra of RNA with Na^+ ions on ORN-1-coated spots. Two peaks are detected: near *m/z* [3891.0 + nNa^+] and [7782.5 + nNa^+] (Figure 3a). The former peak corresponds to the molecular weight (MW = 3891.2) of ORN-1. The associated molecular weight for the latter peak (2MW + nNa^+) is consistent with the dimer formation by ORN-1. Na^+ ion adducts are clearly observed for the dimeric G-quadruplex as shown in Figure 3b. A relatively high intensity peak for three Na^+ ions indicated that these ions are bound tightly to the G-quadruplex consistent with the cations inside the structures stabilizing the G-quadruplex.[59,60] A little lower intensity at 2 Na^+ may correspond to a single Na^+ loss of three inside Na^+ ions when the structure is disrupted in the gas phase, whereas a 4 Na^+ signal presumably is associated with three inside ions and a related Na^+ ion of the 3′- or 5′-end uridine. Other adducts are easily removed in the gas phase as nonspecific adducts on the phosphate groups. The MALDI-TOFMS data are in excellent agreement with the results obtained from CD and NMR studies and have the further advantage of giving quantitative details on the effects of metal ions. These results suggested that the dimeric G-quadruplex was formed by ORN-1. To the best of our knowledge, this is the first time that an RNA G-quadruplex was directly observed by MALDI-TOFMS.

Gel Electrophoresis Studies on 12 nt Human Telomere RNA Sequence. The gel electrophoresis technique is sensitive to structural conformations and has been extensively used to study G-quadruplex folding.[61–64] We carried out nondenaturing gel electrophoresis to investigate the structure of 12 nt telomeric RNA at concentrations of NaCl ranging from 50 to 200 mM. When salt is absent in the running buffer of gel electrophoresis, the ORN-1 shows a single and slightly slower mobility than the 12 nt control RNA containing the G to A mutations within individual G-tetrads, consistent with the monomeric species (Figure S5). Importantly, we observed that, in the presence of salt, the ORN-1 migrates faster than the single-strand control RNA and exhibits a single electrophoretic band at all concentrations of Na^+, indicating a more compact G-quadruplex formation by ORN-1 even in the low salt concentration. These observations suggested that telomere RNA can form a G-quadruplex structure described above.

Formation of G-Quadruplex Resists Telomere RNA Degradation. Azzalin et al. showed that UUAGGG-containing RNA exists in cells at higher levels than complementary CCCUAA-containing RNA molecules (They did not indicate the reasons).[30] We hypothesized that the high level of UUAGGG-containing RNA may result from its strong RNase resistance induced by G-quadruplex formation. To test this hypothesis, an RNase T1 digestion experiment was performed. This enzyme cleaves RNA specifically on the 3′ side of unpaired G. In the absence of Na^+, more than 90% of ORN-1 was consumed after 5 min of digestion (lane 4 in Figure. 4). In the presence of Na^+, however, almost no consumption was detected (lane 3). Each of the paired

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Figure 4. ORN-1 was digested with RNase T1. Lane 1: without RNase T1, Na^+ and K^+ ions. Lane 2: with T1 and K^+ ions without Na^+ ions. Lane 3: with T1 and Na^+ ions without K^+ ions. Lane 4: with T1 without Na^+ and K^+ ions.

G's in ORN-1 was protected from RNase T1 digestion,[65] suggesting that the telomere RNA is base-paired in the G-quadruplex structure proposed. We noted that the RNase T1 digestion was also inhibited in the presence of K^+ (lane 2). Presumably, telomere RNA forms a higher-order structure in a K^+ solution. These results suggested that G-quadruplex formation induced a strong RNase resistance and resulted in a high-level existence for UUAGGG-containing telomere RNA.

Possible Biological Significance. In contrast to DNA quadruplex structures, RNA quadruplexes are less characterized. Recently, there have been several reports of the participation of RNA quadruplexes in gene regulation.[34,42] The UUAGGG-containing telomere RNA existing at higher concentrations than complementary CCCUAA-containing RNA suggested that G-quadruplex formation by the single-stranded telomeric RNA does not have to compete with hybridization to a complementary strand, implying that the formation of the RNA quadruplex by UUAGGG-containing telomere RNA might be biologically more relevant. The finding of telomere RNA molecules opens new doors to better understanding the essential biological role of telomere. We found that the telomere RNA G-quadruplex induced a strong RNase resistance for UUAGGG repeats telomere RNA. This result agrees with the observation that the repeats RNA exists in cells at higher levels and enriches 8−10 -fold in purified polyadenylated RNA compared to total RNA.[30,31] The telomere RNA G-quadruplex resisting telomere RNA degradation may offer a concentration supply of the telomere RNA to participate in essential biological processes. The proposed structures might also have clinical relevance in the treatment of cancer, as the RNA molecule may contribute to the telomeric alterations accompanying malignant transformation.[31] Thus, such G-quadruplex structures may be a valuable target for anticancer agents directed against telomeres. In this regard, it will be of great interest to evaluate the capacity of known DNA G-quadruplex ligands to bind to equivalent structures in telomere RNA.

The differential stabilizing effect of cations has been proposed as the driving force for an ion-dependent switching mechanism that may control transitions between different nucleic acids structures.[66–69] Many studies have been performed to investigate the effect of Na^+ ions on G-quadruplex structures. For example, Sen and Gilbert suggested that switching between antiparallel and parallel structures may be controlled by the intracellular balance of sodium and potassium ions,[67,68] and these interconversions could be biologically relevant. It has been indicated that Na^+ cations can be coordinated within a tetrad of a G-quadruplex to stabilize the structures,[70] consistent with our MALDI-TOFMS observations that three Na^+ ions at a relatively high intensity may locate within three tetrads of the telomere RNA G-quadruplex. The current study on the telomere RNA structure is the first step in providing insight into the Na^+-coordinated structure of a parallel G-quadruplex adopted by human telomere RNA. Further experimental studies of a K^+-coordinated structure of human telomere RNA is currently under investigation by NMR analysis in our laboratory.

Experimental Section

RNA Synthesis and Purification. RNAs were synthesized with an Applied Biosystems 3400 DNA/RNA synthesizer, using the solid-phase phosphoramidite chemistry and a DMT-OFF cycle. After automated synthesis, the oligomers were detached from the support, deprotected, and purified by HPLC. The oligomers were identified by a matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOFMS) on an autoflex III smartbeam mass spectrometer (positive mode).

CD Measurements and Analysis of CD Melting Profile. CD spectra were measured using a Jasco model J-725 CD spectrophotometer. The spectra were recorded using a 1 cm path length cell. Samples were prepared by heating the oligonucleotides at 90 °C for 5 min and gradually cooling them to room temperature. In CD melting studies, diluted samples were equilibrated at room temperature for several hours to obtain equilibrium spectra. The melting curves were obtained by monitoring a 265 nm CD band. Solutions for CD spectra were prepared as 0.3 mL samples at a 17 μM strand concentration in the presence of 100 mM NaCl, 10 mM Tris-HCl (pH 7.0).

NMR Experiments. NMR experiments were performed on a Bruker DRX-600 spectrometer. An RNA sample of 0.34 mM was dissolved in 0.5 mL of 90% H_2O/10% D_2O, 10 mM Na-phosphate, pH 6.8, 200 mM NaCl. Assignments of the proton resonances were initially made by using the methods previously described for assignments of quadruplex DNA structures.[71,72] These assignments were confirmed by comparison of NOESY spectra to those of the related telomere DNA (Figures S6, S7).[44]

MALDI-TOFMS. The MALDI matrix was 3-hydroxypicolinic acid (HPA), 50:50 acetonitrile (ACN)/H_2O, ammonium iron citrate. 1 μL of RNA sample (0.5 mM, 200 mM NaCl) was mixed with 1 μL of matrix solution. A spot of 1 μL of the sample−matrix mixture was placed on a stainless steel (384-well) MALDI target plate and

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allowed to air dry at room temperature. The MALDI-TOFMS spectrum was measured using a matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOFMS) on an autoflex III smartbeam mass spectrometer (positive mode).

Nondenaturing PAGE Experiments. The RNA samples were heated at 95 °C for 5 min in the presence of 50, 100, and 200 mM NaCl followed by incubation at 4 °C for overnight. Nondenaturing gel electrophoresis experiments were performed in a 0.5 × TBE buffer in the presence or absence of NaCl. The samples were loaded onto a nondenaturing 10% polyacrylamide gel and run for 2 h (8 V/cm) at 4 °C. Gels were stained with GelStar nucleic acid gel stain (TaKaRa). The resulting gel was imaged with an imaging analyzer (FLA-3000).

RNase T1 Digestion. RNA ORN-1 (17 μM strand concentration) was digested with 1 unit RNase T1 in 100 μL aliquots of 150 mM NaCl, 150 mM KCl, or in the absence of Na^+ and K^+ (10 mM Tris-HCl pH 7) for 5 min at 37 °C. The cleaved products were analyzed on a denaturing 20% (w/v) polyacrylamide gel and visualized by an imaging analyzer (FLA-3000).

Acknowledgment. This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture, and Technology of Japan. Support by the Global COE Program for Chemistry Innovation is also acknowledged.

Supporting Information Available: CD measurements (Figures S1, S2, S3), NMR spectra (Figures S4, S7), Nondenaturing gel electrophoresis (Figure S5), and NOESY pattern (Figure S6) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA8031532