Mutagenesis Reveals an Unusual Combination of Guanines in RNA G-Quadruplex Formation

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ABSTRACT: The classic G-quadruplex motif consists of a continuous array of 3–4 guanine residues with an intermittent loop size of 1–7 nucleotides (Gx−N1−Gx−N1−Gx−N1−Gx−N1−). An RNA G-quadruplex is able to attain only one parallel G-quadruplex topology owing to steric constraints. Investigating the possibilities of the formation of RNA G-quadruplexes with a stretch of sequences deviating from this classic motif will add to the overall conformations of RNA G-quadruplexes, bestowing diversity to this structure. Here, we report unusual combinations of guanine residues involved in RNA G-quadruplex formation in the 5′ untranslated region (UTR) of the von Willebrand factor (VWF) mRNA using the mutagenesis approach. Different permutations and combinations of guanine residues form G-quadruplexes. Upon investigation, G-quadruplexes in 5′ UTR of VWF mRNA are shown to exhibit an inhibitory function.
RESULTS

Dual Luciferase Assay for Assessing the Role of the G-Rich Sequence in the 5′ UTR of VWF. To assess the role of the G-rich sequence in the 5′ UTR of VWF, the entire 5′ UTR of the VWF gene was cloned at the 5′ end of the Renilla luciferase gene of psiCHECK-2 vector (wtUTR). As a control, a mutant construct mUTR was generated, where guanine residues at several positions were mutated to adenines (mUTR). The presence of the G-rich sequence in the 5′ UTR of the VWF (wtUTR) repressed the reporter gene expression to 59% (Figure 1). This indicates that the G-rich sequence in the VWF UTR executes gene inhibitory function.

Quantification of mRNA Levels in the Presence and Absence of the G-Rich Sequence Using Real-Time Polymerase Chain Reaction. To assess the changes in the transcript level in the presence and absence of the G-rich sequence, real-time polymerase chain reaction (PCR) of HEK 293T cells transfected with wtUTR and mUTR constructs was performed. The transcript levels of reporter genes were similar in cells transfected with wtUTR and mUTR constructs, indicating that the UTR G-rich sequence does not alter the mRNA levels of this gene (Figure 2).

Biophysical Characterization of VwtRNA and VmutRNA Sequences. We took a wild-type G-rich sequence (VwtRNA) of VWF and a mutant version of the same sequence (VmutRNA), where we mutated guanine residues at four positions. We performed UV melting of the VwtRNA and VmutRNA sequences in 10 mM sodium cacodylate buffer with wild-type construct (wtUTR) and mutant construct (mUTR) harboring point mutations (highlighted in yellow color in the above table where the sequences represent a snapshot of 250 nts long 5′ UTR of VWF cloned in psiCHECK-2 vector) in G-rich region of 5′ UTR of VWF. Experiments were performed in triplicate and the results are expressed in mean ± SE. Asterisks indicate statistical significance (Student’s t-test) relative to mUTR-transfected cells (*, p < 0.05).

Figure 1. G-rich sequence in 5′ UTR of VWF mRNA inhibits gene expression. Dual luciferase assay was performed with HEK 293T cells transfected with wild-type construct (wtUTR) and mutant construct (mUTR) harboring point mutations (highlighted in yellow color in the above table where the sequences represent a snapshot of 250 nts long 5′ UTR of VWF cloned in psiCHECK-2 vector) in G-rich region of 5′ UTR of VWF. Experiments were performed in triplicate and the results are expressed in mean ± SE. Asterisks indicate statistical significance (Student’s t-test) relative to mUTR-transfected cells (*, p < 0.05).

Figure 2. The mRNA levels of reporter genes remains unchanged in the presence and absence of G-rich sequence in 5′ UTR of VWF. The transcript level of reporter genes in cells transfected with wtUTR and mUTR constructs were quantified using real-time RT-PCR. Experiments were performed in triplicate, and the results are expressed in mean ± SE.
presence of these ions. We compared the intensity of footprint signals in the presence of K+ versus Mg2+. In the presence of K+ ions, several guanine residues are protected against RNase T1 cleavage, as reflected by the less intense bands on gels (Figure 4). Footprinting of VWF RNA shows protection of guanine residues G15, G17−18, G20−22, G26−28, G30−32, G34−35, and G38−39 (highlighted in red color) in the presence of potassium ions. The numbering of these guanine residues is done on the basis of their position in the 5′ UTR of VWF. The protection of these large number of guanine residues well indicates highly polymorphic G-quadruplex formation in this RNA. It suggests that a mixed population of G-quadruplex structures forms under in vitro condition.

Dual Luciferase Assay for Determining the Number of G-Quartets Involved in G-Quadruplex Formation in the 5′ UTR of Genes. Before determining the complete sequence of the G-quadruplex under cellular conditions, we assessed whether G3 quadruplex (G-quadruplex with three G-quartets) formation is taking place in the VWF gene. Thus, we individually mutated each guanine residue in continuous stretches of three Gs (M1, M2, M3, M4, M5, M6, M7, M8, and M9) by site-directed mutagenesis and examined the changes in gene expression upon cell transfection.

Mutations of the consecutive guanine residues in G-tracts of the G-quadruplex sequence in the VWF 5′ UTR (M1, M2, M3, M4, M5, M6, M7, M8, and M9) brought back the gene expression of these mutants similar to that of control mUTR, where G-quadruplex formation was disrupted (Figure 5). This suggests that these residues are highly important for G-quadruplex activity and show that G3 quadruplex formation occurs in the 5′ UTR of VWF.

Dual Luciferase Assay for Determining Complete G-Quadruplex Sequences in the 5′ UTR of VWF. Once we have shown that the G-quadruplex in the 5′ UTR of VWF is composed of three G-quadets, we pursued our search for the complete G-quadruplex forming sequence in the 5′ UTR of VWF. As we have already traced three G-tracts (comprising three guanine residues), there is a requirement for a fourth G-stem to form G-quadruplexes. Because these G-tracts have G-rich neighbors at both their sides, we initially assessed the role of guanine residues toward the 5′ region. We mutated the guanine residues G17 and G18 in M10 construct and G15 in M11 plasmid, transfected them into cells, and performed dual luciferase assay. We observed that mutations in the M10 and M11 plasmids reduced the gene expression to 32 and 22%, respectively (Figure 6). Surprisingly, this inhibition in reporter
protein production by these plasmids was higher compared to wtUTR, where the presence of G-quadruplexes suppressed the reporter protein production to 59%. This suggested that instead of abrogating G-quadruplex-mediated gene suppression, these mutations have aggravated its suppressive effects. This further indicates that these guanine residues are not participating in the G-quadruplex function under normal conditions. As a matter of fact, it suggested that guanine residues toward the 3′ region of three G-tracts are involved in the G-quadruplex formation.

Thus, to pinpoint the guanine residues involved in the G-quadruplex stem formation, we individually mutated several guanine residues present in the 3′ region of the constructs—M12, M13, M14, M15, M16, M17, and M18. Again, mutations in M15 and M16 caused further reduction in gene expression (31 and 44%, respectively), whereas the Renilla luciferase gene expression in M12 and M13 was similar to that of the wtUTR (Figure 7). Interestingly, the mutations in M17 and M18 increased the gene expression (71 and 82% respectively) as compared to wild-type constructs (59%) and the mutations in M14 brought back the gene expression similar to control plasmid mUTR, where the G-quadruplex formation was completely disrupted. There was no significant difference in

| Mutation | Renilla Relative Expression |
|----------|----------------------------|
| wtUTR    | 1.00                        |
| M12      | 0.69                        |
| M13      | 0.69                        |
| M14      | 0.69                        |
| M15      | 0.31                        |
| M16      | 0.44                        |
| M17      | 1.21                        |
| M18      | 1.21                        |
| mUTR     | 0.59                        |

Figure 5. G-quadruplex in 5′ UTR of VWF mRNA contains three G-quartets. Dual luciferase assay results of HEK 293T cells transfected with wtUTR and constructs with point mutations in G-tracts (highlighted in yellow color in the table above where the sequences represent a snapshot of 250 nts long 5′ UTR of VWF cloned in psiCHECK-2 vector). Experiments were performed in triplicate and the results are expressed in mean ± SE. Asterisks indicate statistical significance (Student’s t-test) relative to VWF mUTR-transfected cells (*, p < 0.05).
the reporter gene expression between M14 and mUTR. These results suggested that guanine residues mutated in M14 (G38) and M18 (G45) constructs are highly important in G-quadruplex formation.

Furthermore, to identify a third guanine residue engaged in the G-quadruplex stem formation, we generated mutants M19, M20, M21, M22, and M23, where we mutated several guanine residues to permit the possibility of only one G-quadruplex formation. We found that the Renilla luciferase expression in M19, M20, M21, M22, and M23 are 46, 35, 37, 81, and 51%, respectively (Figure 8).

Next, we performed UV melting experiments using four mutated oligonucleotides that correspond to M12, M15, M16, and M22 (sequences from 20th base to 45th base as shown in the Figures 7 and 8) along with the wild-type sequence. We observed that the oligonucleotides corresponding to M12, M15, and M16 show typical hypochromicity upon recording absorbance at 295 nm as a function of temperature (Figure 8).
confirming the formation of G-quadruplexes. The oligonucleotide that corresponds to M22 does not show typical hypochromicity, indicating its inability to form G-quadruplexes.

Figure 9. UV melting experiments for the sequences with bases numbered from 20 to 45 shown in WT, M12, M15, M16, and M22. Experiments were performed in 10 mM sodium cacodylate buffer (pH 7.4) containing 100 mM KCl using 2 μM oligonucleotides.

DISCUSSION

In comparison with the DNA G-quadruplex, the RNA G-quadruplex is a newer field of research. Information on RNA G-quadruplex formation is limited to classic G-quadruplex motifs, where the guanine residues in G-stretches vary from 2 to 4 nucleotides. Owing to the presence of steric hindrance induced by the 2’ hydroxyl group of ribose sugars, only parallel topology is possible limiting the conformational diversity of the RNA G-quadruplex. Recently, stacked dimeric G-quadruplexes with bulges have been reported in the 5’ terminal region of human telomerase RNA (hTERC). Another report by Perreault and group showed that an RNA G-quadruplex can form with a long central loop of 30 nucleotides and can modulate translation. Also, RNA mimics containing G-quadruplex structures of unusual topology were shown to induce fluorescence in chromophore.

Although studies on unusual DNA G-quadruplex formation validated their occurrence in in vitro conditions by primarily employing an NMR technique approach, their existence in in cellulo condition still requires authentication. Thus, we added dimensions to our study by examining the existence of unusual RNA G-quadruplexes in cellular conditions and assessing its function in the UTR of an mRNA. We employed site-directed mutagenesis and reporter assay approach to test the guanine residues involved in the RNA structure formation in the 5′ UTR of VWF mRNA.

We explored the role of the G-rich sequence in the 5′ UTR of VWF mRNA by cloning the entire 5′ UTR of VWF gene in reporter vector (wtUTR) and generating mUTR mutant that mutated guanine residues at several positions. On performing the dual luciferase assay, we obtained a significant decrease in the Renilla luciferase gene expression in wtUTR constructs, indicating that the G-rich sequence in the 5′ UTR of VWF mRNA exhibits gene-inhibiting function. Furthermore, the transcript profile of reporter genes in cells transfected with wtUTR and mUTR was similar, indicating that the G-rich sequence does not regulate transcription and mRNA stability.

Furthermore, to test the ability of the G-rich sequence (VwtRNA) in the VWF UTR to form G-quadruplexes, we performed its biophysical characterization using UV and CD spectroscopy in 10 mM sodium cacodylate buffer containing 100 mM KCl. CD spectra of the VwtRNA sequence displayed the parallel G-quadruplex signature, well in agreement with the previous reports on RNA G-quadruplexes. Also, on performing UV melting at 295 nm wavelength, the VwtRNA sequence displayed a hypochromic shift with increasing temperature, a trait distinctive to the G-quadruplex. On the other hand, mutRNA mutant sequences harboring mutations in guanine residues did not display CD, TDS, and UV signature of the G-quadruplex, indicating its inability to form G-quadruplexes.

Close inspection of the CD profile (Figure 3B) revealed small amplitude of signals around 290 nm. A UV TDS (Figure 3C) also showed a negative peak at around 290 nm. Additionally, we observed mild hypochromicity below 60 °C in UV melting studies (Figure 3A). All of these observations hint toward the formation of antiparallel structures from a small fraction of the oligonucleotide. So far, all known naturally occurring RNA G-quadruplexes are parallel stranded. However, recently an unusual G-quadruplex motif is found in a 97 nt RNA fluorogenic aptamer known as Spinach. Spinach, an in vitro-selected RNA aptamer that binds a GFP-like ligand and adopts an elongated fold containing two sets of coaxially stacked helical stems that flank the ligand-binding region (L). This L region folds into a new RNA quadruplex motif that contains only two layers of G-tetrads. There are two strands in this L region, and each strand contributes four Gs to the quadruplex, forming two parallel segments via double-chain reversal loops. Within the 5′ side of the ligand binding region, there are two pairs of consecutive residues with antiglycosidic conformations, each of which forms a corner of the quadruplex connected through the double-chain reversal loop. Within the 3′ side of the ligand binding region, pairs of noncontiguous guanosine residues form the other two corners of the quadruplex, connected through the double-chain reversal loop and looped-out residues. Within this configuration, three guanosine residues adopt the syn-glycosidic conformation, and one adopts the anticonformation, giving rise to an unusual G-quadruplex topology. We may have a similar kind of situation in the present case; however, high-resolution structural study is required for any confirmative understanding. Once we confirmed the ability of wtRNA sequences to form G-quadruplexes in vitro, we assessed the participation of three G-quartets in G-quadruplex formation (G3 quadruplex) in the UTR by incorporating mutations in G-tracts of G-quadruplexes of the VWF UTR. Point mutations in G-quartets completely disturbed the activity of G-quadruplexes and showed gene expression similar to mUTR, where G-quadruplex activity was completely disrupted. In other words, we demonstrated that G3 quadruplexes prevail in the VWF UTR, where G-quadruplexes are composed of three G-quartets.

We continued our search for complete G-quadruplex sequences in the VWF UTR by assessing the involvement of guanine residues at the neighborhood of these G-tracts. From results based on point mutations and luciferase reporter assay, we found that the mutations of guanine residues at the 5′ side of G-tracts did not abrogate the function of the G-quadruplex, indirectly suggesting that guanine residues present at the 3′ side of G-tracts are highly important in the G-quadruplex formation. As three guanine residues of the fourth strand of the G-quadruplex are required to form from seven guanine residues, 35 combinations of guanine residues are possible. By employing the procedure of site-directed mutagenesis and reporter assay,
we ascertained the essentiality of two residues: G38 (mutated in M14) and G45 (mutated in M18) in G-quadruplex formation. G38 mutation in M14 restored the gene expression similar to that of the mutant mUTR and G45 mutation in M18 partially rescued the reporter gene expression from the suppressive effects of G-quadruplexes. There is also a possibility of the involvement of M17 (G42) in the G-quadruplex formation, as its mutation slightly increased the gene expression.

Although G34/G35/G39/G41 mutations (M12, M13, M15, M16) did not abrogate the G-quadruplex formation, we hypothesized that one of these residues is involved in the G-quadruplex formation because they lie close to two residues G38 and G45, which are highly important for the G-quadruplex formation. Because the 5′ UTR of VWF is G-rich and the vicinity of three G-tracts contains several guanine residues, the chances of polymorphic G-quadruplex formation are quite high in this region. Different combinations of guanine residues may give rise to the G-quadruplex structure. This polymorphism is well-supported by RNase T1 footprinting results of VWF RNA oligonucleotides, where a large number of guanine residues was protected against cleavage, indicating that mixed population of G-quadruplexes exists under in vitro condition. For this reason, our in cellulo study testing the complete G-quadruplex forming sequence in the VWF 5′ UTR was highly intriguing. Whenever we mutated a guanine residue (a potent candidate) to adenine in a few instances, it changed the gene expression of the reporter genes in other directions. Instead of restoring the reporter gene expression back to the mutant mUTR, it further repressed the gene expression. Point mutations in this G-rich region probably permit the other guanine residues to take up the charge of the G-quadruplex formation, demonstrating a shift in the equilibrium upon mutating guanine residues. The resulting G-quadruplex function deviates from the wild-type G-quadruplex role and may modify the extent of gene regulation accordingly. As a matter of fact, as point mutations change the gene expression of the reporter gene extensively, we speculate that a specific combination of G-tracts within this segment may aid the formation of a conformational switch, thereby fine-tuning the VWF gene expression. The fine-tuning of the VWF gene expression is of prime importance because it is involved in blood clot formation and homeostasis maintenance. The existence of such a flexible conformational switch has previously been reported in mRNA G-quadruplexes located in VEGF IRES.16

Owing to the shift in equilibrium in overall G-quadruplex configuration, it was very difficult to pin-point a third guanine residue involved in G-quadruplex formation under normal cellular conditions. However, as we have identified the involvement of G38 and G45 residues in the G-quadruplex, it has narrowed down our search for the third residue from five possible combinations. To further identify the third guanine residue among G34, G35, G39, G41, and G42 forming the G-quadruplex stem, we undertook a reverse approach where we negated the possibility of polymorphic G-quadruplex formation and permitted the chance of only one G-quadruplex formation involving G38, G45, and G34/G35/G39/G41/G42 (M19, M20, M21, M22, M23). However, dual luciferase assay of all of these mutants showed G-quadruplex existence and inhibitory function, indicating the possibility of G34/G35/G39/G41/G42 involved in the stem formation. Among these mutants, M23 mutant showed gene expressions similar to that of wild-type wtUTR, suggesting that the G-quadruplex formed in M23 may be mimicking the activity of G-quadruplexes present in the VWF wtUTR.

In UV melting studies, we observed that oligonucleotide corresponding to M22 did not show any typical hypochromicity upon recording absorbance at 295 nm as a function of temperature, indicating its inability to form G-quadruplexes, which in fact was also reflected its inability to suppress the protein expression in luciferase studies. Mutations in M22 increased the gene expression to 81%. Oligonucleotides corresponding to M12, M15, and M16 showed typical hypochromicity, confirming the formation of G-quadruplexes and corroborating qualitatively with observations obtained in luciferase studies. However, M12 that affects the luciferase expression similarly as wtUTR gives a very different melting profile from wtUTR. Oligonucleotides deviating from a typical

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Figure 10. Probable models of G-quadruplex structure in 5′ UTR of VWF mRNA. The numbering of guanine residues represents their position in 5′ UTR of VWF mRNA.
**MATERIALS AND METHODS**

**Oligonucleotides.** The HPLC-purified RNA oligonucleotides (Table 1) were purchased from Sigma-Aldrich.

| oligonucleotide name | sequence |
|----------------------|----------|
| VwtRNA               | GGGAAAGGGAGGGUGUUGGUGGAUG |
| VmutRNA              | GAGAAAGAGAGAGAGUUUGUGGAUG |

**Scheme 1. Schematic Representation of 5′ UTR of VWF Cloned at the Nhe I Site of Renilla Luciferase Gene of psiCHECK-2 Vector**

"The position of forward primer (FP) and reverse primer (RP) used for cloning is also shown. The G-rich sequence in 5′ UTR of VWF is highlighted in bold.

**Biochemical Studies**

Table 1. Details of RNA Oligonucleotides Used for Biophysical Studies

G3_{1-}N1_{1-}G3_{1-}N1_{1-}G3_{1-}G3_{1-}sequence need some kind of an inducer to form a quadruplex in vivo. We have seen that the in vitro-selected RNA aptamer25 does not have typical G-stretches in its sequence and does not give typical quadruplex signature in UV melting, CD, and footprinting at low concentrations. However, at very high concentrations (mM range) and in the presence of the GFP-like ligand, this aptamer showed quadruplex signature in vitro. Similarly, here we speculate that some cellular components, such as proteins, can act as inducers to form highly stable RNA quadruplexes. Actually, it is highly difficult to correlate in vivo and in vitro results in the absence of inducers. More similar type of studies is required to have a fair knowledge on inducers and their role in RNA quadruplex formation.

The best strategy to test the unusual RNA G-quadruplex occurrence is to extrapolate the findings of DNA G-quadruplexes at the RNA level. “Alternate G-quadruplex with bulge” is the structure known to exist in DNA, where the G-stretch interfering nucleotide forms the bulge projecting out of the G-quadruplex core.4 The RNA G-quadruplex was revealed to form from the sequence GGGUUGGGAGGGGUGGGG, where cytosine-interrupting contiguous guanine residues form a bulge employing NMR studies.27 On the basis of these reported alternate G-quadruplex structures and our results, we built five models of alternate RNA G-quadruplexes present in the 5′ UTR of VWF in normal cellular conditions. All of these models show that the residues G20–22, G26–28, G30–32, G38, and G45 are involved in the stem formation and residues A23–A25, A29, and U33 are involved in the loop formation. Out of these, four models have two bulges and one model has only one bulge. In the first model, G34 is involved in the stem formation; U33 is involved in the loop formation; the residues G35, U36, and U37 form the first bulge; and G39, U40, G41, G42, A43, and U44 form the second bulge (Figure 10A). In the second model, G35 forms the stem; U33 and G34 form the loop; U36 and U37 form the first bulge; and G39, U40, G41, G42, A43, and U44 form the second bulge (Figure 10B). In the third model, G39 is involved in the stem formation; U33, G34, G35, U36, and U37 form the loop; and the residues U40, G41, G42, A43, and U44 form the bulge (Figure 10C). In the fourth model, G41 constitutes stem; U33, G34, G35, U36, and U37 are involved in the loop formation; G39 and U40 form the first bulge; and G42, A43, and U44 form the second bulge (Figure 10D). In the fifth model, G42 constitutes the stem; U33, G34, G35, U36, U37 form the loop; the residues G39, U40, G41 constitute the first bulge; and A43 and U44 constitute the second bulge (Figure 10E).

To unravel the overall structure of such G-quadruplexes under in vitro condition, NMR of VWF RNA is highly required. As the existence of the unusual structure in DNA G-quadruplexes was demonstrated using the NMR approach, the same would highlight the residues involved in RNA G-quadruplex formation; however, it would be difficult to experimentally establish this complicated fold, particularly for RNA (personal communication with Prof. Anh Tuan Phan). Moreover, in vitro studies would not be able to build an overall picture of an unusual RNA G-quadruplex at the cellular level as indicated by RNase T1 footprinting analysis.

To summarize, our study pioneers in establishing the existence as well as the importance of the unusual RNA G-quadruplex structure in the UTR of mRNA. The existence of RNA G-quadruplex structures with varied combinations of guanine residues requires extensive research, and much progress on its functional aspects is called for. Testing the alternative ways to form G-quadruplexes will diversify the entire arena of RNA G-quadruplex complexity, predictability, and functionality.
M20, M21, M22, and M23) were carried out using a Kappa Ready Mix (Saflabs). Briefly, wild-type constructs (wtUTR) were PCR-amplified with primers containing mutations at the annealing temperature of 58 °C. Then, the wild-type vector backbone was digested with Dpn I at 37 °C for 6 h. DH5α Escherichia coli cells were then transformed by these PCR products. The positive clones obtained were confirmed by sequencing.

**Cell Culture.** The HEK 293T cell line was maintained at the humidified atmosphere containing 5% CO₂ incubator at 37 °C. The cells were grown in high-glucose DMEM supplemented with 10% fetal bovine serum and antibiotic–antimycotic (Gibco).

**Dual Luciferase Assay.** The HEK 293T cells in a 24-well plate were transfected with the aforementioned plasmids using the lipofectamine 2000 reagent (Invitrogen). The dual luciferase assay was performed after 48 h of incubation. Briefly, the transfected cells were lysed with passive lysis buffer. Then, the Renilla and firefly luciferase readings of cell lysates were obtained using a dual luciferase assay kit (Promega) and a Tecan plate reader. The ratio of the Renilla luciferase activity to the firefly luciferase activity was subsequently calculated and normalized against the corresponding mutant plasmid readings (mUTR).

**Quantitative Real-Time PCR.** The HEK 293T cell lines were transfected with the plasmids (wtUTR and mUTR) in a 48-well plate using the lipofectamine 2000 reagent (Invitrogen). The dual luciferase assay was performed after 48 h of incubation. Briefly, the transfected cells were lysed with passive lysis buffer. Then, the Renilla and firefly luciferase mRNA levels were quantified using real-time PCR. Briefly, the total RNA of the transfected cells was isolated using the Trizol reagent. Then, cDNA was prepared using the MMuLV reverse transcriptase enzyme and oligo dT primers (Fermentas). Subsequently, forward and reverse primers of the firefly and Renilla luciferase genes were used to quantify mRNA levels of reporter genes using a SYBR-Green PCR Master Mix (Applied Biosystems) and the Roche Detection system. The transcript level of Renilla luciferase gene was normalized using the Pfiabl method and depicted in histogram.

**UV Spectroscopy.** The RNA samples (2 μM) were prepared in 10 mM sodium cacodylate buffer (pH 7.4) containing 100 mM KCl. The samples were first preheated at 95 °C for 5 min and then slowly cooled to 4 °C. The UV melting experiments were performed using a Cary 100 UV–vis spectrophotometer (Varian) equipped with a Peltier temperature controller. The samples were heated at 95 °C and then allowed to cool to 20 °C at 0.2 °C/min. The absorbance values were recorded at the wavelength of 295 nm and at 0.2 °C data intervals of heating and cooling steps. The UV melting of VWTRNA was also performed at different strand concentrations in 10 mM sodium cacodylate buffer (pH 7.4) containing 100 mM KCl.

**Circular Dichroism.** The RNA samples (2 μM) were prepared in 10 mM sodium cacodylate buffer (pH 7.4) containing 100 mM KCl. The CD experiments were conducted in a Jasco J-810 spectropolarimeter (Jasco Hachioji, Tokyo, Japan) equipped with a Peltier temperature controller, and scans were taken at 200–350 nm wavelength at 20 °C. Postsubtraction of buffer alone, the CD scans were taken in triplicate and the resultant average scan was collected.

**Thermal Difference Spectrum.** TDS is calculated by recording the absorbance of RNA samples at the wavelength of 200–350 nm at temperatures 20 and 95 °C. The TDS curve is then obtained by subtracting their absorbance values and plotting it against the wavelength. The RNA samples were prepared in 10 mM sodium cacodylate buffer (pH 7.4) containing 100 mM KCl.

**In Vitro Transcription.** The dsDNA template for IVT was prepared by annealing the primer set at 37 °C for 1 h.

The RNA was transcribed from the DNA template harboring T7 promoter using a MEGAscript Kit (Ambion). Briefly, the DNA template was incubated with NTPs, T7 DNA polymerase, and enzyme mix buffer for a duration of 16 h at 37 °C. The template DNA was then degraded using the TURBO DNase enzyme (Ambion). The RNA products were subsequently purified using NucAway columns (Ambion).

**Footprinting.** RNA oligonucleotides were radiolabeled using a KinaseMax kit (Ambion). Briefly, RNA was incubated with T4 polynucleotide kinase and (γ-32P) ATP for 1 h at 37 °C. The reaction was stopped using 1 mM ethylenediaminetetraacetic acid (EDTA) and by heating at 95 °C for 2 min. The labeled RNA was further purified using NucAway columns (Ambion).

5′ end radiolabeled RNAs were heated at 90 °C for 5 min and subsequently cooled to 37 °C in 1 mM MgCl₂, 150 mM KCl, and 150 mM LiCl to assist the structure formation. The structured RNA was then digested with 0.025 units of RNase T1 for 15 min at 37 °C. In addition, an RNase T1 ladder was generated by digesting RNA in buffer containing 20 mM Tris HCl (pH 7.5), 10 mM MgCl₂, and 100 mM LiCl for 2 min at 37 °C with 0.6 U of RNase T1. All reactions were stopped by the addition of stop buffer containing 95% formamide and 18 mM EDTA and subsequent immediate snap chilling on dry ice. Equal counts of digested products were separated onto a 15% denaturing gel in 0.5× TBE containing 18% acrylamide and exposed to a phosphorimager screen. The gel images were scanned on a Typhoon scanner (GE Healthcare).

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**Notes**

The authors declare no competing financial interest.

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### ABBREVIATIONS

VWF, von Willebrand factor; UTR, untranslated region; CD, circular dichroism; TDS, thermal difference spectrum; Tm, melting temperature

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