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Identification of a Conserved Sequence Motif of an RNA Aptamer Binding to a G-rich Sequence RNA with Structural Probes

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Guanine-rich sequences observed in terminal segments of eukaryotic genomes[1,2] have the potential to form the non-canonical four-stranded topology called G-quadruplexes, which are built from the stacking of successive GGGG tetrads and stabilized by bound monovalent Na^+ and K^+ cations.[3] They appear to play a critical role in cellular aging and cancer.[4-8] The end of telomeric DNA decreases in length after each round of cell division in somatic cells.[9] But the telomere length can be maintained by the enzyme telomerase, a ribonucleoprotein complex with reverse transcriptase activity in most cancer cells.[10] The guanine-rich sequences, putative G-quadruplex-forming elements in the 5'-UTRs of the human genome have been indentified.[11] One of these sequences, an 18-mer containing four guanine-tracts, 5'-GGGAGGGGCGGGUCUGGG-3' is associated with the 5'-UTR of the oncogenic N-ras sequence. According to a cell-free translation system coupled to a reporter gene assay, the N-ras G-quadruplex can inhibit gene expression at the translational level.[11] This result indicates that molecules stabilizing 5'-UTR RNA G-quadruplex formation can be the candidates for therapeutic agents, thereby inhibiting the translation of the oncogene.

SELEX is a technique for isolating nucleic acid molecules (aptamers) with affinities for a target molecule from a random pool with a large number of sequences by the iterative rounds of affinity selection and amplification. Target molecules include proteins, amino acids, nucleotides, antibiotics and RNA.[12-24] We isolated RNA molecules binding to the guanine-rich RNA (Fig. 1) in the 5'-UTR RNA of N-ras oncogene from an RNA pool.[25] After the 11th round of *in vitro* selection, the sequences of the selected RNA aptamers were closely related and had the consensus sequence GGGAUCCGCAUGCAAGCUUA.[26] This sequence is thought to be important to the interaction between the selected RNA aptamer and the ligand G-rich sequence RNA. The selected RNA aptamers can recognize the specific domain of RNA structure like a monoclonal antibody and be candidates of the anticancer agent at the genetic level. So it is important to determine the structure of the selected RNA aptamers and to get the information for the interaction between an RNA aptamer and a ligand RNA used for selection. In this study, the secondary structure of a selected RNA aptamer was determined with RNA structural probes such as RNase T1 and RNase V1 and the conserved sequence

(A) 5′-GGGAGGGGCGGGUCUGGG-3′

(B) **GGGAUCCGCAUGCAAGCUUA**CCGACUGCGCGU

Figure 1. (A) Sequence of guanine-rich RNA used for selection and (B) sequence of the randomized region in RNA aptamer 11-30-24. The consensus sequence is underlined in bold letters.

motif for the interaction between an RNA aptamer and a ligand G-rich sequence RNA was identified.

The secondary structure of 11-30-24 (Fig. 1), one of RNA aptamers selected from an RNA pool including the constant primer sequences, is predicted and shown in Figure 2. This structure was found by the CLC RNA workbench ver. 4.2 program accessed on the internet (www.clcbio.com). The biochemical experiments to investigate the predicted secondary structure of the RNA aptamer were performed by using RNase T1 that has the specificity for a guanine in single-stranded region and RNase V1 that is double-strand-specific. Nucleotides G12, G16, G23, G39 and G43 were cleaved by RNase T1, suggesting that these guanines are thought to be in single-stranded region of the secondary structure model of RNA aptamer 11-30-24. G8, G27, G29, G31, G33 and G34

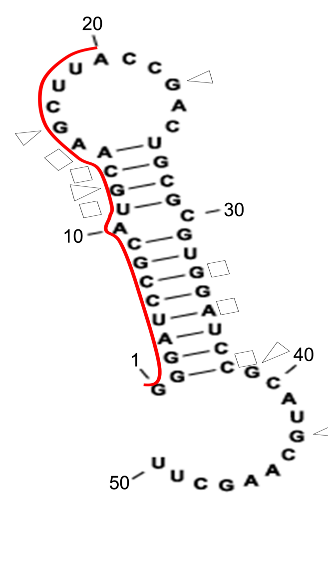

Figure 2. Schematic representation of possible secondary structures of RNA aptamers 11-30-24. Triangles indicate the sites cleaved by RNase T1 and squares indicate the sites cleaved by RNase V1.

were not cleaved by RNase T1 so theses guanines are thought to be in double-stranded region. The fact that nucleotide G12 which is thought to be in double-stranded region was weakly digested by RNase T1 suggests that base pair G12:C28 is not stable. The accurate identification for this base pair can be obtained after studying the tertiary structure of RNA aptamer 11-30-24. On the other hand, nucleotides U11, C13, G33, A35 and C38 were clearly cleaved by RNase V1, suggesting that these nucleotides are located in double-stranded region. Interestingly, A15 which is thought to be in single strand by RNase T1 treatment, was also susceptible to RNase V1 treatment. It is likely that this nucleotide may be stacked from intramolecular interaction and became accessible to RNase V1 in solution. In summary of the secondary structure of RNA aptamer 11-30-24, it has one long double-stranded region flanked by single-stranded loop and single strand at the 3'-terminus. The double-stranded region of RNA aptamer is made up with 12 base pairs and two bulges. The bulges A10 and C30 were found in the double strand. It has the large single-stranded loop with eleven nucleotides (A15GCUUACCGAC25). In conclusion, the conserved sequence motif, G1GGAUCCGCAUGCAAGCUUA20 is extended in the single- and double-stranded region in the secondary structure model.

In order to discover potential regions participating in the interaction between the G-rich sequence RNA and RNA aptamer 11-30-24 we have performed footprinting experiments using RNase T1 and RNase V1 (Figure 3). Nucleotides G12, G16 and G23 in RNA aptamer 11-30-24 became more susceptible to RNase T1 in the presence of G-rich sequence RNA (R-G) than the RNA aptamer alone (R). This observation suggests that these nucleotides were liberated from the intramolecular interaction and became more accessible to

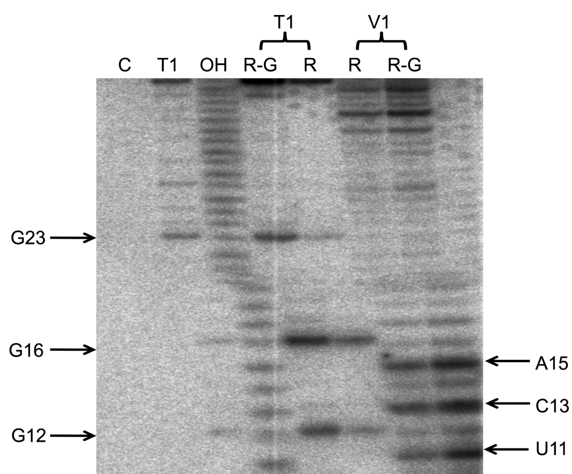

Figure 3. Enzymatic probing of the interaction between the guanine-rich sequence RNA and RNA aptamer 11-30-24. RNA aptamer labeled at the 5'-end was partially digested with RNase T1 and RNase V1 in the presence (R-G) or absence (R) of the guanine-rich sequence RNA. Aliquots of enzymatic digestions were loaded onto a denaturing 15% polyacylamide gel, along with the corresponding partial-alkaline hydrolysate (OH), the denaturing partial RNase T1 (T1) and no digestion (C). The protected nucleotides are indicated by arrows.

RNase T1 in solution upon forming a complex with G-rich sequence RNA. On the other hand, nucleotides U11, C13 and A15 in RNA aptamer 11-30-24 became more susceptible to RNase V1 in the presence of G-rich sequence RNA than the RNA aptamer alone, suggesting that these nucleotides were stacked or located in double-stranded region from the intramolecular interaction and became more accessible to RNase V1 in solution upon forming a complex with G-rich sequence RNA. In summary, a sequence motif conserved among the selected RNA aptamers contains a sequence (U11GCAA15) identified from a footprinting assay and is thought to play an important role to the interaction between these two RNAs. Further study is needed to know the nature of the interaction between the guanine-rich RNA and RNA aptamer -whether the interaction between these two RNAs occurs through Watson-Crick base-pairing or a specific tertiary interaction.

Experimental Section

SELEX Protocol. The affinity column for *in vitro* selection was prepared as described.[22] In brief, guanine-rich RNA was synthesized by run-off *in vitro* transcription with T7 RNA polymerase from the DNA template to which the T7 promotor was annealed and purified by PAGE (polyacrylamide gel electrophoresis) and gel elution of the crush and soak method.[27] The gel-purified guanine-rich RNA was oxidized at the 3'-terminal sugar with $NaIO_4$ and then coupled to Sepharose-adipic acid hydrazide resin (Amersham Pharmacia Biotech). Selection was performed with the guanine-rich RNA-attached column. In order to minimize the enrichment of undesirable RNA species binding to the Sepharose resin itself, we pre-selected the RNA pool on the uncoupled Sepharose-adipic acid hydrazide resin. After being passed through the pre-column of Sepharose-adipic acid hydrazide resin, RNA library was passed through the affinity column and then RNA species bound to the column were eluted by reducing the ionic strength and chelating the Mg^{2+} with EDTA. In other words, we loaded the RNA pool onto the guanine-rich RNA-attached affinity column. We washed the column with binding buffer and then eluted the bound RNA with three column volumes of the elution buffer (25 mM Na-EDTA, pH 8.0). We recovered the selected RNAs by ethanol precipitation and reverse-transcribed it with an M-MLV reverse transcriptase (RT) (bioneer, Korea) (or AMV reverse transcriptase, promega) using a cDNA primer (5'-AAGCTTGCATGCGGATCC-3'). Then, the cDNAs were amplified by PCR with the cDNA primer and T7 primer (5'-TAATACGACTCACTATAGGTG-3'). A new pool of RNA, enriched in the guanine-rich RNA-binding motifs, was prepared by transcription from the PCR-amplified DNA and used for the next round of selection and amplification. The stringency of the selection was given to lead to a more cohesive sets of RNA isolates by reducing the concentration of the guanine-rich RNA to make the affinity column as the number of selection cycle increased. After the 11th round of selection, the amplified cDNAs were cloned into the pGEM-

T Easy vector (Promega), and their sequences were determined.

Enzymatic Protection Experiment. RNA aptamer 11-30-24 was synthesized by run-off *in vitro* transcription with T7 RNA polymerase from the DNA template to which the T7 promotor was annealed and purified by PAGE and gel elution of the crush and soak method. The purified RNA was treated with CIP (calf intestinal alkaline phosphatase) to remove 5' terminal phosphate and then labeled at 5'-end with [γ-32P]ATP (GE Healthcare) and T4 polynucleotide kinase (New England Biolab). The 5' end radiolabeled RNA was purified by PAGE and renatured. The labeled RNA alone or mixed with the non-labeled cognate RNA was used for nuclease-cleavage reactions. The reaction volume was adjusted to 100 μL with binding buffer (30 mM Tris-acetate, pH 7.5, 60 mM magnesium acetate, 120 mM potassium acetate, and 120 mM ammonium acetate) and incubated for 20 min at room temperature. Then, 1 U of RNase T1 (Industrial Research Limited) or 0.1 U of RNase V1 (Pierce Molecular Biology) was added to the above mixture. The reaction mixture was incubated for another 20 min at room temperature. The cleavage products were recovered by ethanol precipitation and electrophoresed on 15% polyacrylamide- 7 M urea gel.

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