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Notes

Identification of Structural Motif of RNAs Interacting with the G-rich Sequence RNA

Bongrae Cho

Department of Applied Chemistry, Cheongju University, Cheongju 360-764, Korea
*E-mail: brcho@cju.ac.kr*

Guanine-rich tracts are observed in the terminal segments of eukaryotic genomes.[1,2] These guanine-rich sequences have the potential to form the non-carnonical four-stranded topology called G-quadruplexes. The G-quadruplexes are built from the stacking of successive GGGG tetrads and stabilized by bound monovalent Na^+ and K^+ cations.[3] The G-quadruplexes play a biological role in DNA telomere ends, the purine-rich DNA strands of the oncogenic promoter elements such as c-myc and c-kit, and RNA 5'-untranslated region (UTR) in close to translational start sites. Telomeres located at the ends of eukaryotic chromosomes are composed of the tandem DNA repeats of guanine-rich sequences and essential for chromosome stability. They appear to play a critical role in cellular aging and cancer.[4-8] Telomeric DNA ends decrease 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 expressed in most cancer cells.[10] So telomeres and telomerases correlated with cancer progression and have been used for the targets of anti-cancer agents.

Attention has been paid to DNA quadruplexes and their potential role in biology. On the other hand, RNA quadruplexes have got less attention, despite the implication of their involvement at the site of translational control. The guanine-rich sequences, putative G-quadruplex-forming elements in the 5'-UTRs of the human genome have recently identified.[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. This sequence is located 14-nucleotides downstream of the 5'-cap and 222-nucleotides upstream of the translation start site. The measured t_m of this sequence was 63 °C in 1 mM K^+ cation and the stabilization decreased in the order K^+ > Na^+ > Li^+. 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 oncogenes.

The expression of genetic information in RNA can be regulated by designing DNA or RNA oligonucleotide complementary to the target sequence. For example, antisense oligonucleotides were developed to specifically repress the complementary target genes.[12,13] But the down regulation of specific mRNA with antisense oligonucleotide needs to be studied further because mRNAs have diverse conformers. To overcome this structural problem, SELEX (Systematic Evolution of Ligands by Exponential Enrichment) was applied to isolate RNA aptamers which specifically bind to the N-ras G-quadruplex RNA in this study.

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.[14-26] In this study, RNA molecules binding to the guanine-rich RNA in the RNA 5'-UTR of N-ras oncogene, were selected from an RNA pool containing 48 randomized nucleotides. An RNA pool for SELEX was prepared by *in vitro* transcription using T7 RNA polymerase from the corresponding double-stranded DNA library of about 10^{14} independent sequences. Selection was performed with the guanine-rich RNA-attached column which was prepared by coupling the 3'-terminal sugar of guanine-rich RNA to the 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
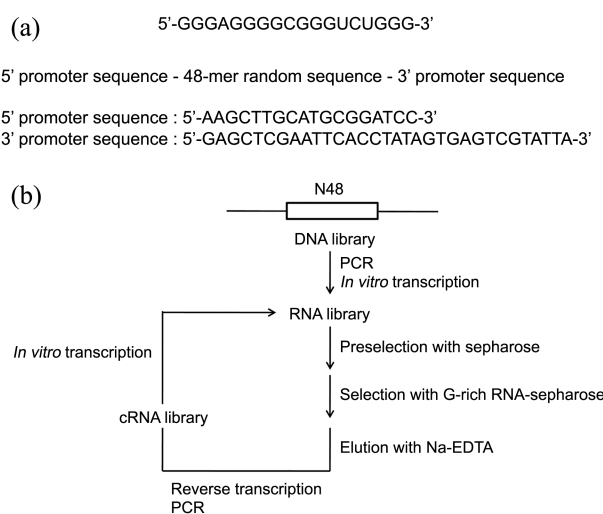
Figure 1. (a) The guanine-rich sequence used for selection and a synthetic library containing 48 randomized nucleotides bound by constant T7 promoter and 3' primer. (b) Outline of the experimental strategy for SELEX. RNA aptamers binding to a guanine-rich sequence RNA were selected with the guanine-rich sequence RNA-attached affinity column chromatography.

11-21-8   **GGGAUCCGCAUGCAAGCUUA**GCGUUGCUGUG

11-21-30 **GGGAUCCGCAUGCAAGCUUA**GGUCGUGUG

11-21-33 **GGGAUCCGCAUGCAAGCUUA**GUUCGCGUUGG

11-30-36 **GGGAUCCGCAUGCAAGCUUA**CUGCCACGUUG

11-48-9   **GGGAUCCGCAUGCAAGCUUA**GUGGCCGU

11-21-39 **GGGAUCCGCAUGCAAGCUUA**GCGUGGGGUUAAUACGACUCACUAUAG

11-48-35 **GGGAUCCGCAUGCAAGCUUA**GUCGAUCGUUAAUACGACUCACUAUAG

Figure 2. Sequences of the randomized region in RNA aptamers. The consensus sequences are underlined in bold letters.

strength and chelating the Mg^{2+} with EDTA. The selected RNAs were recovered, reverse-transcribed, and amplified by PCR. 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. 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 sequences of the selected RNA aptamers were shown in Figure 2. The sequences of two selected RNA aptamers were closely related. We searched the consensus sequences of two selected RNA aptamers with RNA aptamers selected from an RNA pool containing 30 randomized nucleotides using CLUSTAL W(1.83) multiple sequence alignment. The selected RNA aptamers had the consensus sequence GGGAUCCGCAUGCAAGCUUA (Figure 2). This consensus sequence was also observed in RNA aptamers selected from an RNA pool containing 30 randomized nucleotides .
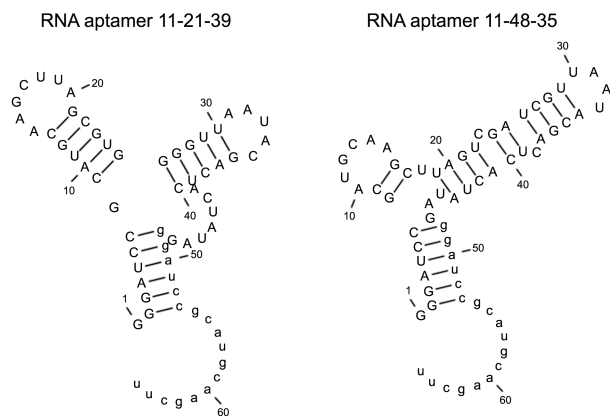
Optimal predicted secondary structures of two RNA aptamers, 11-21-39 and 11-48-35, selected from an RNA pool containing 48 randomized nucleotides including the constant sequences 5' and 3' to the 48 nt sequence, are shown in Figure 3. These structures were found by the CLC RNA workbench ver. 4.2 program accessed on the internet (www.clcbio.com). Both RNA aptamer 11-21-39 and 11-48-

Figure 3. Predicted secondary structures of RNA aptamers 11-21-39 and 11-48-35. Primer sequences at the 5' and 3' ends of the transcript are indicated in lower case and unique portions of the molecules are indicated in capital letters.

35 have two stem-loop regions and one double-stranded region separated by single strands. On the whole, they have secondary structure similar to the cloverleaf secondary structure found in tRNA. Conserved sequence motif, GGGAUCCGCAUGCAAGCUUA was extended in the single- and double-stranded region in the secondary structure models. Now the secondary structures of RNA aptamers predicted with theoretical method, the CLC RNA workbench ver. 4.2 program, are biochemically been examining with RNA structural probes such as RNase T1 that has the specificity for a guanine in single strand region, double-strand-specific RNase V1 and single-strand-specific nuclease S1.

Experimental Section

Construction of a Random RNA Library. The guanine-rich sequence in the RNA 5'-UTR of N-ras oncogene used in this study, was shown in Figure 1(a). The oligonucleotide library, 5'-AAGCTTGCATGCGGATCC-(N)48-GAGCTCG AATTCACCTATAGTGAGTCGTATTA-3', contained 48 randomized nucleotides flanked by primer binding site at 5' side and the promoter sequences at 3' side, respectively (Figure 1(a)). This library was amplified through five cycles of polymerase chain reaction (PCR). The library of 1 μg was transcribed *in vitro* with T7 RNA polymerase (RiboMAX™, Promega, Madison, WI, USA) in 20 uL of the reaction buffer. *In vitro* transcription products were purified by gel elution of the crush and soak method.[27] The gel-purified RNA was dissolved in 0.2 mL of the binding buffer (30 mM Tris-acetate, pH 7.5, 60 mM magnesium acetate, 120 mM potassium acetate, and 120 mM ammonium acetate), incubated at 70 ºC for 5 min, and slowly cooled to room temperature.

SELEX Protocol. The affinity column for *in vitro* selection was prepared as described.[24] In brief, guanine-rich RNA was purified by gel elution from *in vitro* transcribed products. 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). 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, we then 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'), and used for the next round of selection and amplification. After the 11th round of selection, the amplified cDNAs were cloned into the pGEM-T Easy vector (Promega),

and their sequences were determined.

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