http://dx.doi.org/10.5012/bkcs.2013.34.6.1924

Conserved Sequence Motif of RNA Aptamers Binding to the G-rich Sequence RNA

Bongrae Cho

Department of Applied Chemistry, Cheongju University, Cheongju 360-764, Korea. E-mail: brcho@cju.ac.kr
Received March 14, 2013, Accepted March 27, 2013

Key Words : Sequence motif, G-rich sequence RNA, 5'-UTR, RNA aptamer, SELEX

Guanine-rich tracts are observed in 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] They 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) 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] 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 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 of 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 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. This sequence is located in 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 the oncogene.

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] We used SELEX technique to get the information for the RNA-RNA interaction and got the similar results with affinity chromatography and gel mobility shift assay. According to this result, we knew that similar results could be got irrespective of SELEX techniques employed.[21] We have already isolated RNA molecules binding to the guanine-rich RNA in the 5'-UTR RNA of N-ras oncogene from an RNA pool containing 48 randomized nucleotides.[27] So in this study to know that similar result for the RNA-RNA interaction can be got irrespective of numbers of random nucleotides in RNA pool, RNA molecules binding to the same guanine-rich sequence RNA were selected from another RNA pool containing 30 randomized nucleotides and compared with the previous result from an RNA pool

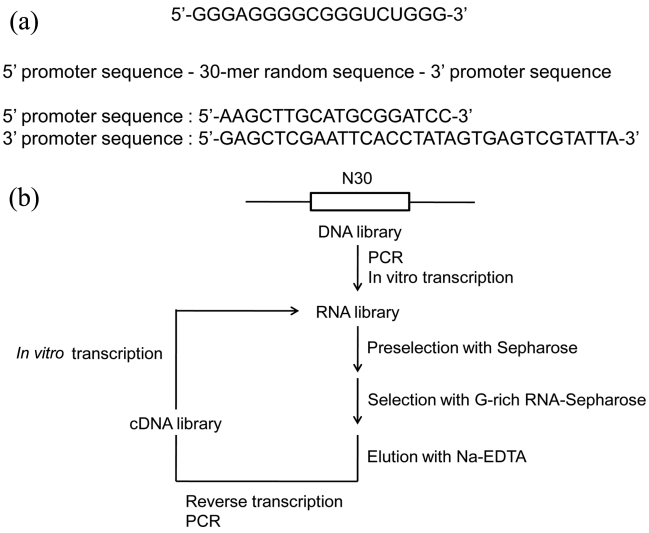

Figure 1. (a) The guanine-rich sequence used for selection and a synthetic library containing 30 randomized nucleotides bound by constant T7 promoter and 3' primer. (b) Outline of the experimental strategy for SELEX. RNA aptamers binding to a guanine-rich sequence RNA were selected with the guanine-rich sequence RNA-attached affinity column.

11-21-32 **GGGAUCCGCAUGCAAGCUUA**CAGCCGGUGU
11-48-2 **GGGAUCCGCAUGCAAGCUUA**CAGCCGGUGU
11-48-17 **GGGAUCCGCAUGCAAGCUUA**CAGCCGGUGU
11-48-37 **GGGAUCCGCAUGCAAGCUUA**CAGCCGGUGU
11-48-40 **GGGAUCCGCAUGCAAGCUUA**CAGCCGGUGU

11-21-5 **GGGAUCCGCAUGCAAGCUUA**CUGCUCGUGUG
11-21-31 **GGGAUCCGCAUGCAAGCUUA**CUGCUCGUGUG
11-48-4 **GGGAUCCGCAUGCAAGCUUA**CUGCUCGUGUG

11-30-31 **GGGAUCCGCAUGCAAGCUUA**CCGACUGCGCGU
11-30-24 **GGGAUCCGCAUGCAAGCUUA**CCGACUGCGCGU
11-48-34 **GGGAUCCGCAUGCAAGCUUA**CCGACUGCGCGU

11-30-32 **GGGAUCCGCAUGCAAGCUUA**CACUCGGCGU
11-48-41 **GGGAUCCGCAUGCAAGCUUA**CACUCGGCGU

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

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

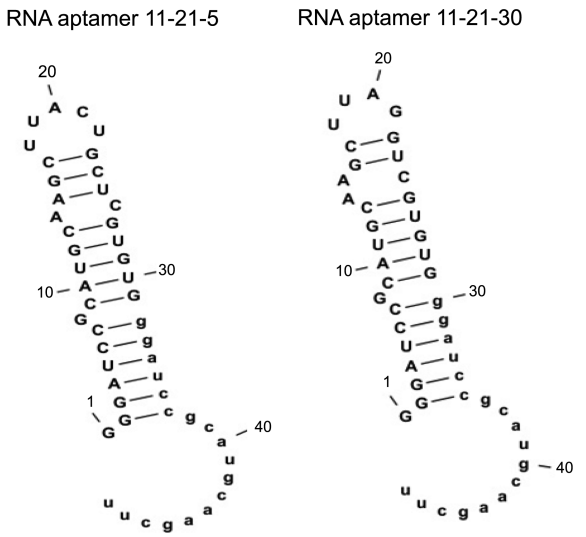

Figure 3. Predicted secondary structures of RNA aptamers 11-21-5 and 11-21-30. Primer sequences of the transcripts are indicated in lower case and unique portions of the molecules are indicated in capital letters.

containing 48 randomized nucleotides. After the 11th round of *in vitro* selection, the sequences of the selected RNA aptamers were shown in Figure 2. The sequences of selected RNA aptamers were closely related and several sequences have identical sequences. For example, RNA aptamer 11-21-5 was found as three identical sequences and RNA aptamer 11-48-2 was found as five identical sequences. We searched the consensus sequences of the selected RNA aptamers with CLUSTAL W(1.83) multiple sequence alignment. The selected RNA aptamers had the consensus sequence GGGAUCC GCAUGCAAGCUUA (Figure 2). This consensus sequence was also observed in RNA aptamers already selected from an RNA pool containing 48 randomized nucleotides.[27] So this sequence is thought to be important to the interaction between the selected RNA aptamer and the ligand G-rich sequence RNA.

Optimally predicted secondary structures of two RNA aptamers selected from an RNA pool containing 30 randomized nucleotides, 11-21-5 with three identical sequences and 11-21-30 with only one identical sequence, including the constant primer sequences to the 30 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 11-21-5 and 11-21-30 have one long double-stranded region flanked by single-stranded loop and single strand. Double-stranded region of RNA aptamer 11-21-5 and 11-21-30 is made up with 14 nucleotides and 13 nucleotides, respectively. And bulges were found in the double-stranded region. Bulges G8, A14 and C26 were found in the double-stranded region of RNA aptamer 11-21-5 and bulges G8, A14A15 and C24 were found in the double-stranded region of RNA aptamer 11-21-30. RNA aptamer 11-21-5 has five nucleotides in single-stranded loop and RNA aptamer 11-21-30 has four nucleotides in the same loop. Conserved sequence motif, GGGAU CCGCAUGCAAGCUUA was extended in the single- and double-stranded region in the secondary structure models. The biochemical experiments to investigate the secondary structures of RNA aptamers predicted in this study are now underway using RNA structural probes by RNase T1 that has the specificity for a guanine in single strand region, double-strand-specific RNase V1 and single-strand-specific nuclease S1 treatment.

Experimental Section

Construction of a Random RNA Library. The guanine-rich sequence in the 5'-UTR RNA of N-ras oncogene used in this study, was shown in Figure 1(a). The oligonucleotide library, 5'-AAGCTTGCATGCGGATCC-(N)30-GAGCTCG AATTCACCTATAGTGAGTCGTATTA-3', contained 30 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). 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. In other words, the library of 1 μg was transcribed *in vitro* with T7 RNA polymerase (RiboMAX^{TM}, 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.[28] 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 °C 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). 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'-AAGCTT GCATGCGGATCC-3'). Then, the cDNAs were amplified by PCR with the cDNA primer and T7 primer (5'-TAATA CGACTCACTATAGGTG-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.

Acknowledgments. I thank Prof. Younghoon Lee (Department of Chemistry and Center for Molecular Design and Synthesis, Korea Advanced Institute of Science and Technology) for his helpful support.

References

1. Williamson, J. R. *Ann. Rev. Biophys. Biomol. Struc.* **1994**, *23*, 703.
2. Krauskopf, A.; Blackburn, E. H. *Ann. Rev. Genet*. **2000**, *34*, 331.
3. Gellert, M.; Lipsett, M. N.; Davies, D. R. *Proc. Natl. Acad. Sci. USA* **1962**, *48*, 2013.
4. Wright, W. E.; Tesmer, V. M.; Huffman, K. E.; Levene, S. D.; Shay, H. W. *Genes Dev.* **1997**, *11*, 2801.
5. Greider, C. W. *Curr. Opin. Genet. Dev.* **1994**, *4*, 203.
6. Blackburn, E. H. *Cell* **1994**, *77*, 621.
7. Rhodes, D.; Giraldo, R. *Curr. Opin. Struct. Biol*. **1995**, *5*, 311.
8. Verdun, R. E.; Karlseder, J. *Nature* **2007**, *447*, 924.
9. Sfeir, A. J.; Chai, W.; Shay, J. W.; Wright, W. E. *Moll. Cell* **2005**, *18*, 131.
10. Freider, C. W.; Blackburn, E. H. *Cell* **1985**, *43*, 405.
11. Kumari, S.; Bugaut, A.; Huppert, J. L.; Balasubramanian, S. *Nat. Chem. Biol*. **2007**, *3*, 218.
12. Stein, C. A.; Krieg, A. M. *Applied Antisense Oligonucleotide Technology*; Stein, C. A., Krieg, A. M., Eds.; Wiley: New York, 1998.
13. Crooke, S. T. *Annu. Rev. Med.* **2004**, *55*, 61.
14. Giver, L.; Bartel, D. P.; Zapp, M. L.; Green, M. R.; Ellington, A. D. *Gene* **1993**, *137*, 19.
15. Sassanfar, M.; Szostak, J. W. *Nature* **1993**, *364*, 550.
16. Burgstaller, P.; Kochoyan, M.; Famulok, M. *Nucleic Acids Res*. **1995**, *23*, 4769.
17. Binkley, J.; Allen, P.; Brown, D. M.; Green, L.; Tuerk, C.; Gold, L. *Nucleic Acids Res*. **1995**, *23*, 3198.
18. Lato, S. M.; Boles, A. R.; Ellington, A. D. *Chem. Biol*. **1995**, *2*, 291.
19. Lauhon, C. T.; Szostak, J. W. *J. Am. Chem. Soc.* **1995**, *117*, 1246.
20. Geiger, A.; Burgstaller, P.; von der Eltz, H.; Roeder, A.; Famulok, M. *Nucleic Acids Res*. **1996**, *24*, 1029.
21. Cho, B.; Taylor, D. C.; Nicholas, H. B., Jr.; Schmidt, F. J. *Bioorg. Med. Chem*. **1997**, *5*, 1107.
22. Haller, A. A.; Sarnow, P. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 8521.
23. Wallace, S. T.; Schroeder, R. *RNA* **1998**, *4*, 112.
24. Ko, J.; Cho, B.; Ahn, J. K.; Lee, Y.; Park, I. *Bull. Korean Chem. Soc*. **1999**, *20*, 1335.
25. Ko, J.; Lee, Y.; Park, I.; Cho, B. *FEBS Lett*. **2001**, *508*, 300.
26. Cho, B. *Bull. Korean Chem. Soc*. **2005**, *26*, 2093.
27. Cho, B. *Bull. Korean Chem. Soc*. **2012**, *33*, 2796.
28. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.