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단신 (Notes)

SELEX with Affinity Chromatography on RNA-RNA Interaction

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INTRODUCTION

RNA-RNA interaction is a feature of many biological processes including translation, post-transcriptional regulation, RNA interference and the action of ribozymes. The interaction between RNA and RNA can occur through normal Watson-Crick interaction or specific tertiary interaction. The expression of genetic information in RNA can be regulated by designing DNA or RNA oligonucleotide complementary to the target sequence. For example, antisense oligonuclotides were developed to specifically repress the complementary target genes.[1,2] 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, systematic evolution of ligands by exponential enrichment (SELEX) has been applied to isolate RNAs which bind to various kinds of target RNA in our lab.[3–11]

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, antibiotics and nucleotides, etc.[12–20] The oligonucleotide library contains randomized nucleotides, flanked by primer binding site at 5' side and the promoter sequences at 3' side, respectively. The separation of RNA aptamers from RNA library has mainly been achieved in two ways: affinity column chromatography and electrophoretic mobility shift assay. Affinity chromatography has been widely used for the variety of targets owing to simplicity and efficiency since it was demonstrated in 1990.[21–23] The application of SELEX for the RNA-RNA interaction with affinity chromatography was first demonstrated in our study. In this study, we describe the practical procedures for the separation of the RNA aptamers binding to RNA target molecules with affinity chromatography using the target RNA-attached column.

RESULTS AND DISCUSSION

The synthetic library used in this study, is shown at *Fig*. 1 and contain 30 or 48 randomized nucleotides bound by constant T7 promoter and 3' primer. The separation of RNA aptamers from RNA library was achieved with affinity chromatography using the column attached with the target RNA. Target RNA molecules for SELEX as shown in *Table* 1 were *E. coli* 5S rRNA, yeast $tRNA^{Phe}$, *E. coli* $tRNA^{Val}$, a stem-loop RNA at the gag-pol junction of HIV-1 RNA and a guanine-rich sequence in the 5'-UTR RNA of N-ras oncogene. For the preparation of RNA-attached affinity column, the RNA target molecules were synthesized by transcription from the template DNAs with the T7 promoter and purified by gel elution. The gel-purified RNA target molecules were oxidized at the 3'-terminal sugar with $NaIO_4$ and then coupled to Sepharose-adipic acid hydrazide resin. The coupling efficiency of these RNA targets to resin was observed to be more than 95%.[24] During the SELEX with affinity chromatography, 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, the RNA pool was loaded on the RNA target molecule-attached affinity column. Predominant RNA families were obtained in the binding popula-

5′ promoter sequence – random sequence – 3′ promoter sequence

5′ promoter sequence : 5′-AAGCTTGCATGCGGATCC-3′
3′ promoter sequence : 5′-GAGCTCGAATTCACCTATAGTGAGTCGTATTA-3′

***Figure* 1.** A synthetic library containing randomized nucleotides bound by constant T7 promoter and 3' primer.

***Table* 1.** Target RNAs and characterization of SELEX

Target RNA	Number of randomized nucleotides	Number of SELEX cycles	Conserved sequences in selected RNA aptamers
E. coli 5S rRNA	48	12	GCGG, GUGAAA, GUUCAUA
Yeast $tRNA^{Phe}$	48	12	CGAUC, GUAUGGAGAAU, GUGUA
E. coli $tRNA^{Val}$	48	14	CGAAC, GAAGU, GUUCCCUUAG, GUGUA, CGACGG, ACAGU
Stem-loop RNA	30	13	AAGGG, GUUGAU
Guanine rich RNA	30(or 48)	11	GGGAUCCGUAUGCAAGCUUA

tion through 11−14 rounds 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 target RNA to make the affinity column as the number of selection cycle increased. The binding activity of the selected RNA aptamers to target RNA was characterized by 15% polyacrylamide gel electrophoresis under native condition.[4–6] And the nature of the interaction between selected RNA aptamer and target RNA was confirmed to be a specific tertiary interaction rather than normal Watson-Crick base-pairing, according to the result that the deoxyoligonucleotides which have identical sequences with the sequences conserved among selected RNA aptamers did not bind to target RNA on gel mobility-shift assay.[9]

RNA aptamers binding to RNA target molecules could briefly be characterized as follows. First, RNA aptamers binding to *E. coli* 5S rRNA were selected from an RNA pool containing 48 randomized nucleotides. After the 12th round of SELEX, some of the selected RNA aptamers had the consensus sequences GCGG and GUGAAA, which are found in G688CGG691 of 16S rRNA and GUUCAUA, which are found in the loop region of helix 89 of 23S rRNA.[5,6] Second, RNA aptamers binding to yeast $tRNA^{Phe}$ were selected from an RNA pool containing 48 randomized nucleotides. After the 12th round of SELEX, some of the selected RNA aptamers had the consensus sequences C40GAUC44 which is found in 5S rRNA, G1114UAUGG1119 which is found in yeast 18S rRNA, and A2613GAAU2617 and G2609UGUA2613 which are found in yeast 28S rRNA.[8] Third, RNA aptamers binding to *E. coli* $tRNA^{Val}$ were selected from an RNA pool containing 48 randomized nucleotides. After the 14th round of SELEX, some of the selected RNA aptamers had the consensus sequences C43GAAC47 which is found in 5S rRNA, G1491AAGU1495 and G1379UUCC1383 which are found in 16S rRNA, and C1064UUAG1068, G2110UGUA2114, C2480GACGG2485 and A2600CAGU2604 which are found in 23S rRNA.[7] From these results, we came to get the important information for the interaction between tRNA and rRNAs in protein synthesis.

Fourth, RNA aptamers binding to the stem-loop RNA which plays an important role in the synthesis of the gag-pol fusion protein of HIV-1, were selected from an RNA pool containing 30 randomized nucleotides. After the 13th round of SELEX, some of the selected RNA aptamers had the consensus sequences AAGGG and GUUGAU.[9] Fifth, RNA aptamers binding to the guanine-rich sequence in the 5'-UTR RNA of N-ras oncogene were selected from an RNA pool containing 30 and 48 randomized nucleotides. After the 11th round of SELEX, some of the selected RNA aptamers had the consensus sequence GGGAUCCG-CAUGCAAGCUUA.[10,11] From these results, we knew that the identical RNA sequences could be selected irrespective of numbers of random nucleotides in RNA pool. The selected RNA aptamers binding to the stem-loop RNA for the synthesis of the gag-pol fusion protein of HIV-1 and the guanine-rich sequence in the 5'-UTR RNA of N-ras oncogene were thought to be the candidates of anti-viral or anti-cancer agents at genetic level.

In summary, RNAs capable of binding to RNA motifs could be obtained with the performance of SELEX using affinity chromatography and SELEX was thought to be a valuable method to get the information for the interaction between two RNAs.

EXPERIMENTAL SECTION

We carried out all procedures at room temperature unless otherwise specified.

Preparation of a Random RNA Library

RNA library was synthesized by run-off *in vitro* transcription with T7 RNA polymerase from the DNA template to which the T7 promotor was annealed. RNA samples was dissolved with RNA loading buffer (Heating the RNA samples at 90 °C for 5 min prior to loading the gel helped dissolve insoluble debris, which could produce retardation in the lanes (revealed when illuminated with hand UV lamp).), vortexed, spinned down, and loaded on 8% PAG. The gel was electrophoresed at 250 V till the dye front

(from the BPB dye in the samples) reached the middle of the gel. Following electrophoresis, the gel was wrapped with plastic film, placed on the plate coated with silica gel (fluorescence UV254, Whatmann) and illuminated with the short wavelength (254 nm) of hand UV lamp (UVP, Upland, CA, USA). The dark band corresponding to RNA was excised with razor, put into a 1.5 mL plastic tube and purified by the gel elution of the crush and soak method.[23] The gel was left to air-dry for 10 min (It was not easy to crush the wet gel with 1.5 mL sealed blue tip because the gel was slippery. Therefore, it was important to dry the gel for 10 min.). The narrow end of 1.5 mL blue tip was sealed with alcohol lamp and the dried gel was crushed with sealed tip (We crushed the gel slice until it was sticky. It usually took 5 or 10 min.). The gel extraction buffer (10 mM Tris-Cl, pH 8.0, 0.3 M NaCl, 1 mM EDTA) equal to or more than gel volume was added, vortexed and incubated at 37 °C for 30 min. The solution was centrifuged at 13,000 g for 1–2 min and the supernatant was collected in new 1.5 mL plastic tube. This collecting step was repeated twice more (We found that it was best to repeat this step three times according to the collecting experiment with labeled RNA. It was hard to collect RNA from the gel even in the more performance of collecting experiment.). The equal volume of phenol:chloroform (24:1) was added to the collected solution, vortexed and centrifuged at 13,000 g for 5 min. We collected the aqueous, upper layer, added 200 μg glycogen as carrier, 0.1 vol of 3 M NaOAc, pH 5.2 and 3 vol of ethanol and left at −70 °C for 20–30 min. RNAs was collected by centrifugation at 4 °C for 10 min.

Preparation of Affinity Column

We dissolved the gel-purified RNA target molecule (~150 μg) in 100 μL of 0.1 M K-phosphate, pH 8.0, added 50 μL of freshly prepared, ice-cold 20 mM $NaIO_4$ and kept the solution on ice in the dark for 2 h (To avoid exposing light to reaction tube, we kept a 1.5 mL plastic tube wrapped with aluminum foil.). The 3'-terminal oxidized RNA was recovered by ethanol precipitation and redissolved in 0.1 mL of 0.1 M K-acetate, pH 5.0. The required amount of Sepharose-adipic acid hydrazide resin (Amersham Pharmacia Biotech) stored at −20 °C was taken, centrifuged and the supernatant was removed. And we added the binding solution to the centrifuged resin, vortexed, centrifuged and discarded the supernatant. We repeated this equilibrium process three times before use. We coupled the oxidized RNA to 0.5 mL of Sepharose-adipic acid hydrazide resin at 4 °C overnight, with gentle mixing. We centrifuged, discarded the supernatant and suspended the collected resin in binding buffer (The absorbance of the supernatant was measured with UV260 nm and the coupling efficiency was detected.). We filled 5.0 mL syringe (0.5–1 mL bed volume) with Sepharose-adipic acid hydrazide resin itself to make an uncoupled column and 5.0 mL syringe (0.5–1 mL bed volume) with RNA-attached Sepharose-adipic acid hydrazide resin to make an affinity column.

SELEX Protocol

We loaded the gel-purified RNA pool on the uncoupled column of Sepharose-adipic acid hydrazide resin in binding buffer (30 mM Tris-acetate, pH 7.5, 60 mM magnesium acetate, 120 mM potassium acetate, and 120 mM ammonium acetate) (This step helps to minimize the enrichment of undesirable RNA species binding to the Sepharose-adipic acid hydrazide resin itself.) and collected the RNA pool. The pool was applied to the affinity column of RNA-coupled resin. The affinity column was washed with 5 mL of the binding buffer and the bound RNA population was eluted with three column volumes of the elution buffer (The buffer owing to its low ionic strength compared with the binding buffer, was able to elute the bound RNAs from the affinity resin.) (*Fig*. 2). The selected RNA was recovered by ethanol precipitation using 200 μg glycogen as carrier. The selected RNA was reverse-transcribed using an M-MLV reverse transcriptase (RT) (bioneer, Korea) (or AMV reverse transcriptase, promega) with 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 target RNA-binding motifs, was prepared by transcription from the PCR-amplified DNA and used for the next round of selection and amplification.

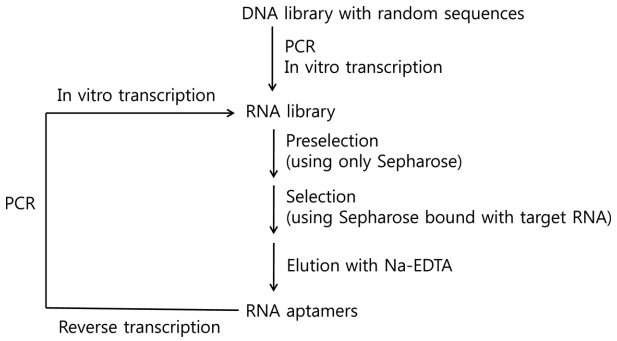

***Figure* 2.** Outline of the experimental strategy for affinity chromatography selection. The target RNA is oxidized at the 3'-end with $NaIO_4$ and bound to Sepharose adipic acid hydrazide. The population capable of binding to the target RNA is eluted from the column by reducing the ionic strength and $[Mg^{2+}]$.

After the 11−14th round of selection, the amplified cDNAs were cloned into the pGEM-T easy vector (Promega) and their sequences were determined.

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