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Effective isolation of RNA aptamer through suppression of PCR bias

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ABSTRACT

An aptamer is a short RNA or DNA molecule that binds to a specific target. The main strategy for obtaining aptamers is systematic evolution of ligands by exponential enrichment (SELEX). Although various SELEX techniques have been devised and refined on the basis of the selection technique used, in most cases, the isolation of an aptamer still requires several trials or the use of special equipment. In the present study, we attempted SELEX in which PCR bias was suppressed by using RNA transcription to amplify nucleic acids. This procedure, which can be accomplished easily and inexpensively without special equipment, effectively simplifies the SELEX process. Using this SELEX, we obtained large numbers of RNA aptamers against the target that could not be isolated by standard SELEX. The results of our study suggest that exclusion of PCR bias may be far more important than previously assumed for isolating RNA aptamers via SELEX.

Introduction

An aptamer is a short RNA or DNA molecule that specifically binds to a target molecule. Systematic evolution of ligands by exponential enrichment (SELEX) is the principal strategy for obtaining aptamers [1]. Many aptamers have been obtained by SELEX; however, the isolation of aptamers by SELEX is not entirely efficient. Although various SELEX techniques for obtaining specific aptamers have been devised [1–6], in most cases, the aptamer production by SELEX still requires several trial-and-error attempts or the use of special equipment [2–5]. Often, only a few clones in a final round of SELEX will bind to a target, while other clones may completely lack affinity for the target. Thus, it becomes necessary to screen many clones in the final round. It is unusual to obtain numerous aptamer clones that include a conserved sequence essential to target binding, although such clones are theoretically contained in the initial library; that is, 100 pmol of the library with a random 40-base region may contain 1.78×10^9 molecules of clones with an identical sequence consisting of 10 bases (see Discussion). Many sequences that are essential to target binding consist of fewer than 10 bases [7–12]. Thus, if both selection and amplification in SELEX were ideal, the isolation of aptamers should theoretically be sufficiently accurate.

During SELEX, purified nucleic acids are amplified by PCR. However, except in the early cycles, clones are not uniformly multiplied by PCR, and an unequal multiplication of clones may decrease efficacy of selection. The T7 RNA polymerase-based RNA amplification method has previously been used to increase a trace of RNA with less amplification bias [13]. Replacement of the majority of PCR amplification cycles with T7 RNA polymerase amplification might decrease amplification bias and increase the precision of selection.

In the present study, we isolated RNA aptamers bound to polyhistidine-tagged macrophage migration inhibitory factor (His-MIF) using a SELEX in which nucleic acids were amplified by transcription (SELEX-T) using T7 RNA polymerase, thereby suppressing the bias of PCR amplification. SELEX-T does not require special equipment, and furthermore, it simplifies the procedure because it does not have a complicated purification of PCR products. After round 7 of SELEX-T, almost all clones tested included a conserved sequence and were bound to the target at a low dissociation rate. It is therefore reasonable to assume that SELEX-T increases the efficiency of SELEX.

Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; His-MIF, polyhistidine-tagged macrophage migration inhibitory factor; SELEX, systematic evolution of ligands by exponential enrichment; SELEX-T, SELEX in which nucleic acids are amplified by transcription.

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Materials and methods

Materials. His-MIF was obtained from ATGen Co. Ltd. (Gyeonggi-do, South Korea). Heat-resistant T7 RNA polymerase was obtained through a ScriptMAX Thermo T7 Transcription Kit from Toyobo Co. Ltd. (Osaka, Japan). The OneStep RT-PCR Kit was obtained from QIAGEN GmbH (Hilden, Germany).

RNA library. Antisense oligonucleotides for the RNA library consists of 40 random nucleotide sequences inserted between two fixed 20 nucleotide sequences and T7 promoter sequences (5′-ACTGCACGTCCAGGCACTGA N_{40} TGAGCGTACGTGAGCGTCCC TATAGTGAGTCGTATTA-3′). The RNA library was transcribed from 50 pmol of antisense oligonucleotides (3×10^{13} molecules) and 250 pmol of T7 promoter primer (5′-TAATACGACTCACTATA-3′) using heat-resistant T7 RNA polymerase.

Biacore analysis. The binding of RNA to the target was measured by an analysis based on surface plasmon resonance using Biacore X (GE Healthcare UK Ltd., Buckinghamshire, UK). RNA plus a poly(A_{20}) tail was immobilized via 5′-biotinylated oligo(dT_{20}) onto the sensor tip with streptavidin (GE Healthcare UK Ltd.). A flow cell without 5′-biotinylated oligo(dT_{20}) was used as the reference cell. After the immobilization of approximately 700 resonance units of RNA, His-MIF was injected as an analyte with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2) containing 100 mM NaCl, 0.01% Tween-20 and 0.1 mM magnesium acetate.

SELEX-T. The conditions for SELEX-T are summarized in Table 1. The RNA library was pre-absorbed with 20 μL of TALON Metal Affinity Resin (Takara Bio Inc., Shiga, Japan) in 50 μL of 10 mM HEPES buffer (pH 7.2) containing 100 mM NaCl, 0.01% Tween-20, and 0.1 mM magnesium acetate with 0.4 U/mL RNase inhibitor and 500 μg/mL tRNA. The non-adsorptive RNA was separated by a membrane filter (Ultrafree-MC Durapore PVDF 5.0 μm, Millipore, Billerica, MA) and incubated for 15 min at 22 °C with His-MIF. The RNA–His-MIF complex was adsorbed with 2.5 μL of TALON Metal Affinity Resin, washed, and then eluted with buffer containing 150 mM imidazole. RNA was purified from the eluate by phenol–chloroform extraction and ethanol precipitation using Ethachinmate (Wako, Osaka, Japan). The purified RNA was transformed into cDNA, including the T7 promoter, with the OneStep RT-PCR Kit (10 μM primers [5′-TAATACGAC TCACTATAGGGACGCTCACGTACGCTCA-3′ and 5′-ACTGCACGTCCAG GCACTGA-3′] at 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min). PCR products were collected by ethanol precipitation and used as the template for RNA production. The RNA was transcribed with heat-resistant T7 RNA polymerase, treated with DNase I, gel-filtrated with Micro Bio-spin 30 Columns (Bio-Rad Laboratories Inc., Hercules, CA), and then purified by phenol–chloroform extraction and ethanol precipitation.

Sequencing. RNA amplified in round 6 or 7 was converted to cDNA using the OneStep RT-PCR Kit (10 μM sequencing primers [5′-TCGAC CTCGAGAAAAAAAAAAGGGACGCTCACGTACGCTCA-3′ and 5′-GAGT CGCGGCCGCTTTTTTTTTTACTGCACGTCCAGGCACTGA-3′] at 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min) and sequenced. RNA amplified in rounds 2–5 was converted to cDNA and sequenced with a Roche Genome Sequencer FLX System (Roche Applied Science, Indianapolis, IN) at Takara Bio Inc.

Results

We measured nonspecific binding of RNA to a target, His-MIF by Biacore analysis (Fig. 1A). Because weak interaction between RNA and the target facilitates the binding and complex formation of an RNA clone with the target, a buffer containing 100 mM NaCl and 0.1 mM magnesium acetate was used in this study.

We hypothesized that the low efficiency of SELEX was a result of PCR amplification bias; namely multiplication of easily amplified clones in PCR was often more predominant than enrichment of desired target-binding clones. To isolate an RNA aptamer against His-MIF, an accomplishment not achieved by standard SELEX, we attempted SELEX-T in which the amplification bias of PCR was suppressed by use of low-cycle PCR with excessive primers and the isolated nucleic acids were mainly amplified by RNA transcription. Using this technique with gentle washing, adequate amounts of RNA were amplified (Table 1). By round 7, the RNAs and all tested RNA clones strongly bound to His-MIF at a very low dissociation rate (Fig. 1B). These results suggested that SELEX-T was practical for isolating RNA aptamers that had a high affinity to target.

As shown in Supplementary Table S1, at round 7, almost all sequenced clones included the conserved sequence [$GGUN_{1-3}$ AYUGGY; Y, pyrimidine base]. All tested RNA clones with the conserved sequence bound to His-MIF (Fig. 1B), whereas some clones without the conserved sequence did not bind to His-MIF (Supplementary Fig. S1). These results suggested that the conserved sequence was important for binding to His-MIF.

The initial RNA library contains 0.137% clones with the conserved sequence (see Discussion). By round 3, the rate of clones with the conserved sequence increased to 50.3%, and the RNA was slightly bound to His-MIF (Table 1); however, a dominant clone was not observed (Table 2). At round 6, a dominant clone represented more than 20% of sequenced clones (Table 2, #701). Finally, by round 7, the clone that bound well to His-MIF, named shot47, increased in number, thereby becoming the second most frequent clone at 10.5% (Table 2). The number of His-MIF bindings per RNA (Table 1), the rate of clones with the conserved sequence (Table 1), and the rate of clones that had a high ability for binding (Table 2), steadily increased round by round. These results suggested that nucleic acid amplification by SELEX-T did not decrease selection and that clones with a high ability for binding could multiply using SELEX-T without exclusion by amplification bias, even though the desired binding sequence was infrequent in the RNA library.

Shot47 bound to the polyhistidine-tag region in His-MIF (data not shown). The details were described in the companion paper.

Table 1
Summary of SELEX-T.

Round	Initial RNA (pmol (μg))	His-MIF (pmol (μg))	Washing (times)	PCR (cycles)	Amplified RNA (μg)	Binding (His-MIF/RNA)	Conserved sequence (%)
1	2500 (65)	300 (5.0)	1	8	44	0.052	Not defined
2	700 (18)	270 (4.5)	2	6	32	0.093	0.840
3	350 (9.0)	140 (2.3)	3	4	8.1	0.35	50.3
4	175 (4.5)	140 (2.3)	3	5	12	0.68	68.8
5	175 (4.5)	140 (2.3)	3	5	17	1.0	82.5
6	175 (4.5)	140 (2.3)	3	5	47	1.2	97.0
7	175 (4.5)	70 (1.2)	4	4	43	1.6	96.2

The number of His-MIF binding to an RNA molecule (Binding [His-MIF/RNA]) was estimated from the amount of molecule on the sensor tip, which was calculated from the signal of surface plasmon resonance (1 pg/mm^2 per resonance unit) [15]. His-MIF binding to RNA library (control RNA, round 0) was 0.10. The RNA library theoretically contains 0.137% of clones, including the conserved sequences (see Discussion).

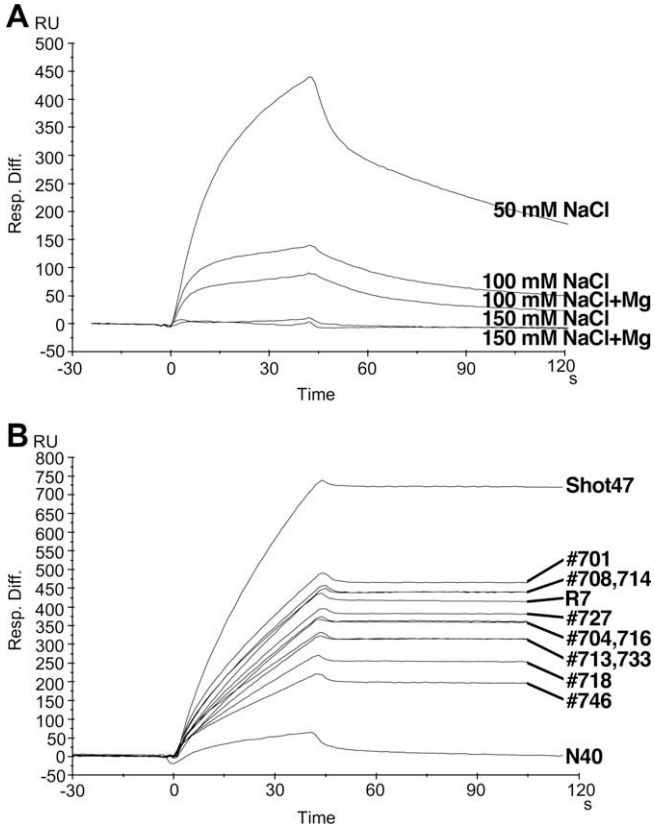

Fig. 1. Binding of RNA aptamers to His-MIF. The binding was measured by Biacore X analysis. (A) Nonspecific binding of RNA to His-MIF. Poly(A)-tailed RNA library was immobilized onto the sensor tip and His-MIF (600 nM) was injected as an analyte with 10 mM HEPES buffer (pH 7.2) containing 0.01% Tween-20 and a series of concentrations of salts. Magnesium acetate was added at 0.1 mM. (B) Binding of isolated RNA aptamers to His-MIF. Each immobilized RNA aptamer plus poly(A_{20}) tail was treated with 600 nM His-MIF in 10 mM HEPES buffer (pH 7.2) containing 100 mM NaCl, 0.01% Tween-20, and 0.1 mM magnesium acetate. The sequence of each clone is shown in Supplementary Table S1. R7 and N40 show RNAs at round 7 and the control RNA, respectively.

Discussion

In comparison to normal RNA, the amplification of RNA aptamers by RT-PCR may be difficult because the RNA aptamer and its cDNA include the sequence derived from the secondary structure through which the RNA aptamer binds to its target. This secondary structure can interfere with RNA transcription or PCR amplification. Since unequal multiplication increases exponentially at each cycle of PCR and each round of SELEX, easily amplified clones, rather than the desired aptamer, may wind up comprising a majority of the sample. By suppressing PCR bias with SELEX-T, we obtained large numbers of RNA aptamers against His-MIF, which we had been unable to isolate under a standard SELEX using PCR with 10–20 amplification cycles. The proportion of each clone was obviously affected by the excessive PCR cycles (Table S2), with the difference increasing round by round. Thus, it is evident that exclusion of PCR amplification bias is much more important for accurately isolating RNA aptamers by SELEX than previously assumed.

The formation of heterogeneous double strand DNA is a source of PCR bias. When a single strand DNA clone anneals with another clone, but not a primer, the clones cannot be duplicated in the cycle. The rate of heterogeneous annealing was a significant issue even under the presence of excessive primer; approximately 10% of sequenced clones were heterogeneous double strands (11 clones per 106 sequenced clones) after 16 cycles of RT-PCR. During SELEX, little PCR product was amplified by PCR using the standard concentration (<1 μM) of primers (data not shown). To suppress heterogeneous annealing, therefore, it is necessary to maintain the concentration of PCR products much lower than that of primers.

Amplification by T7 RNA polymerase is insensitive to this type of interference by reaction products containing transcripts because T7 RNA polymerase increases only primary transcripts without amplification of templates. The proportion of each clone in the RNA population is not significantly influenced by amplification using T7 RNA polymerase [13]. Although RNA transcription is susceptible to interference from a secondary structure of template, a heat-resistant T7 RNA polymerase that can further decrease interference is now commercially available. Since T7 RNA polymerase can replicate >1000 transcripts per template, which is equal to 10 cycles of PCR, the substitution of amplification by RNA transcription for many cycles of PCR would be a reasonable, simple, and effective measure against amplification bias.

In the case of standard SELEX, a minority of clones may often become predominant as RNA aptamers that bind to a target in the final round, while the remaining clones are nonbinding; this had been attributed to the supposition that only a few clones that bound to the target were contained in the library. However, the number of desired clones may be not quite small. Although many oligonucleotide aptamers consist of 20–40 bases, in many cases, the number of bases essential for binding is only about 10 [7–12]. The probability that a specific pattern consisting of 10 bases is present in 40 bases of random sequences is 2.96×10^{-5} [14]. Thus, 100 pmol of a library that includes 40 random bases theoretically contains 1.78×10^{9} molecules of clones with the 10-base pattern. Furthermore, when the binding sequence includes indeterminate bases, the probability of inclusion in the library increases. The probability of the conserved sequence in this study

Table 2
Percentage of representative clones in each round of SELEX-T.

Clone name	Binding (His-MIF/RNA)	R7 (105)	R6 (113)	R5 (6016)	R4 (8431)	R3 (3077)	R2 (8211)
Shot47	1.88	10.5	0.9	0.25			
#701	1.32	*29.5	*20.4	*4.69	0.42	0.16	
#708	1.16	1.9	0.9	0.38	0.09		
#727	1.05	7.6	5.3	3.32	0.52	0.19	
#714	1.01	1.0					
#716	0.99	10.5	6.2	4.02	1.57	0.10	
#704	0.96	5.7	1.8	0.75	0.18	0.03	
#713	0.88	2.9		0.37	0.11		
#733	0.86	1.0	2.7	0.35	0.04		
#718	0.75	1.9	2.7	0.62	0.14		
#746	0.50	1.9	4.4	2.43	*4.47	*1.40	0.01

Sequences of clones are shown in Supplementary Table S1. The total number of clones sequenced in each round is shown in parenthesis. Blank space shows no detection of clone. The * indicates that the clone was dominant in the round.

[GGUN$_{1-3}$AYUGGY; Y, pyrimidine base] was calculated to be 1.37×10^{-3}, which is the sum of the probabilities 4.73×10^{-4} (for GGUNAYUGGY) plus 4.58×10^{-4} (GGUNNAYUGGY) and 4.42×10^{-4} (GGUNNNAYUGGY) [14]. Since innumerable clones with the conserved sequence were isolated by SELEX-T, the bias in the clone proportion isolated by standard SELEX seems to result from the unsuitability of the selection or amplification process in SELEX rather than from a scarcity of the clone in a library.

If there is no oligonucleotide amplification bias, the RNA aptamer that has a high affinity to the target can become predominant by competition after the saturation of RNA aptamer on a target surface. Although RNAs at rounds 3 and 4 were able to bind to a target, there was no dominant clone. After round 5, when the clones with the conserved sequence accounted for >80%, the clones with high affinities to the target became predominant (Tables 1 and 2). These results are consistent with the expectation of competitive selection under no amplification bias, suggesting that the SELEX-T procedure has fewer unknown factors than standard SELEX.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.013.

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