



Evolution of an inhibitory RNA aptamer against T7 RNA polymerase

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ABSTRACT

Aptamers are promising gene components that can be used for the construction of synthetic gene circuits. In this study, we isolated an RNA aptamer that specifically inhibits transcription of T7 RNA polymerase (RNAP). The 38-nucleotide aptamer, which was a shortened variant of an initial SELEX isolate, showed moderate inhibitory activity. By stepwise doped-SELEX, we isolated evolved variants with strong inhibitory activity. A 29-nucleotide variant of a doped-SELEX isolate showed 50% inhibitory concentration at 11 nM under typical *in vitro* transcription conditions. Pull-down experiments revealed that the aptamer inhibited the association of T7 RNAP with T7 promoter DNA.

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1. Introduction

Synthetic gene circuits have applications in the manipulation of biological systems as well as in the generation of simplified models for certain biological phenomena [1–3]. These circuits are constructed by assembling natural, engineered, and/or *de novo* generated gene components. Because synthetic gene circuits have great potential not only for basic biological studies but for industrial and medical applications, efforts are being made to design novel circuit types [1–3].

In nature, RNAs play a wide variety of dynamic roles in intracellular events [4]. These knowledge accumulations have inspired the engineering of artificial gene components based on functional RNAs [5–7]. Compared with proteins, functional RNAs have several attractive features. For example, guide RNAs, including antisense and short interference RNAs, can be designed simply based on base-pair formations. Concerning structural RNAs, the modular properties and the predictability of their two-dimensional (2D) architecture have allowed for rational engineering of these molecules. Furthermore, it is also possible to generate novel, artificial RNAs with desired binding or catalytic properties by employing an *in vitro* methodology [8,9].

RNA aptamers are artificial RNAs that are selected *in vitro* from large random sequence libraries on the basis of their high affinity to target molecules by a process known as SELEX (Systematic Evolution of Ligands by EXponential enrichment) [10–14]. Since the establishment of this procedure, a wide variety of aptamers has been generated, and their applications have been extensively studied [12–14].

In this study, we isolated an inhibitory RNA aptamer against T7

RNA polymerase (RNAP). By virtue of *in vitro* evolution through stepwise doped-SELEX, a small aptamer with strong inhibitory activity was developed. Because T7 RNAP is the most commonly used enzyme for *in vitro* transcription [15–17] and is employable for *in vivo* expression [18–20], the aptamer might be a promising gene component for the construction of synthetic gene circuits.

2. Materials and methods

2.1. SELEX

Preparation of recombinant hexa-histidine-tagged RNAPs and SELEX against T7 RNAP were performed as previously described [21]. The following synthetic DNA was used as an initial DNA pool for SELEX; 5'-AATTTAGGTG ACACTATAGG ATGGCAGCGG AGAG-N35-CAGCCCACAC CACTCTCC-3' (SP6 promoter sequence is underlined, and N35 represents 35-nucleotides [nts] of random sequence). Otherwise mentioned, all synthetic DNAs were purchased from Operon Biotechnologies (Japan).

For the first doped-SELEX, the following synthetic DNA (purchased from Gene Design Inc., Japan) was employed as an initial DNA pool; 5'-AATTTAGGTG ACACTATAGC CCCAGGGATG GGGGAGAGAT ATAGtgtagt gtaagtcaat tctactttcg ttgagccca cACCCTCGG CATCCGTGCC GAG-3' (Uppercase and underlined letters indicate primer-binding sites and SP6 promoter sequence, respectively. Positions indicated by lowercase letters were 15%-doped, that is, mixture of 85% of a base identical to that of the parental T06-38 sequence and 3 × 5% of other bases.) For the second doped-SELEX, the following synthetic DNA (purchased from Gene Design Inc., Japan) was employed as an initial DNA pool; 5'-AATTTAGGTG ACACTATAGC CCCAGGGATG GGGCAAAAGt gtagtGTAAAG TCAAtTcAc tttCgTTGCa gccacATTA CTCGGCATCC GTGCCGAG-3' (Uppercase and underlined letters indicate primer binding sites

Abbreviations: RNAP, RNA polymerase; SELEX, systematic evolution of ligands by exponential enrichment

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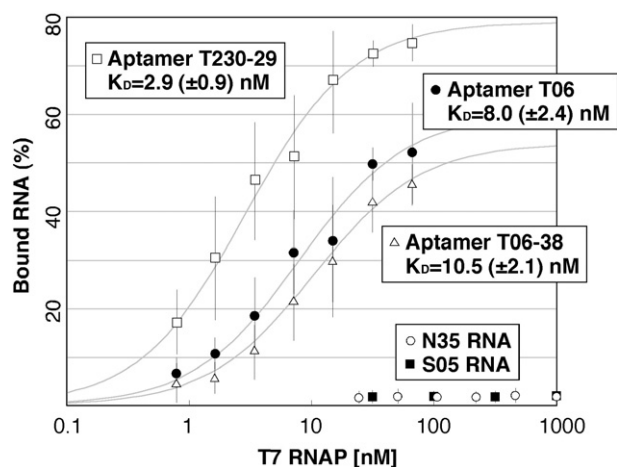


Fig. 1. Estimation of dissociation constant (K_D) by filter-binding assay. Two independent duplicate experiments were performed. Error bars indicate standard deviation. The primary sequences of the aptamers are shown in Table 1.

and SP6 promoter sequence, respectively. Positions indicated by lower-case letters were 60%-doped, that is, mixture of 40% of a base identical to that of the parental T06-38 sequence and $3 \times 20\%$ of other bases). The detailed conditions of the doped-SELEX are described in Supplemental Table 1.

2.2. Biochemical analyses of aptamers

Filter-binding and transcription inhibition assays were performed as previously described [21].

For the evaluation of competition between T7 promoter DNA and aptamer T230-29, 30 pmol of hexa-histidine-tagged T7 RNAP was immobilized onto 3 μ L of HisLink Protein Purification Resin (Promega). The resin was washed two times with buffer P (40 mM Tris-HCl, pH 7.6, 6 mM MgCl₂, 2 mM spermidine, 1 mM DTT, 0.01% BSA, 0.05% NP-40) and re-suspend in 50 μ L buffer P supplemented with 2 units of RNase inhibitor (Takara Bio, Japan). Then, 50 μ L of buffer P containing 10 pmol double-stranded DNA of T7 promoter sequence (Sequence of the non-template strand was 5'-CAAATTAATA CGACTCACTA TAGGGA-3'. The 5'-terminal of the non-template strand was fluorescently labeled with 6-carboxyfluorescein [6-FAM].) and indicated amount of the RNA was added. After 10 min incubation at room temperature, the resin was washed once with 100 μ L of buffer P, re-suspended with 50 μ L of TE buffer, followed by phenol/chloroform extraction. Ten μ L of the water phase was subjected to 10% PAGE, and the recovered DNA was quantified by Storm 840 (GE Healthcare).

3. Results

3.1. Isolation of an inhibitory RNA aptamer against T7 RNAP

Recently, we succeeded in isolating an inhibitory RNA aptamer against SP6 RNAP [21]. To generate an orthogonal pairing of RNAP with an inhibitory aptamer, we intended to isolate inhibitory RNA aptamers against T7 RNAP. SELEX was carried out against hexa-histidine-tagged T7 RNAP using an initial pool containing 3×10^{14} variants of 71-nt RNA with 35-nt random sequences (hereafter, N35 RNA).

After 10 rounds of SELEX, the pool RNA showed increased affinity for and moderate inhibitory activity against T7 RNAP (data not shown). The variants in the pool were cloned, and their inhibitory activities were analyzed. Among 17 clones picked randomly, four clones showed apparent inhibitory activity, and the strongest among these, T06, was chosen for further analyses (Table 1).

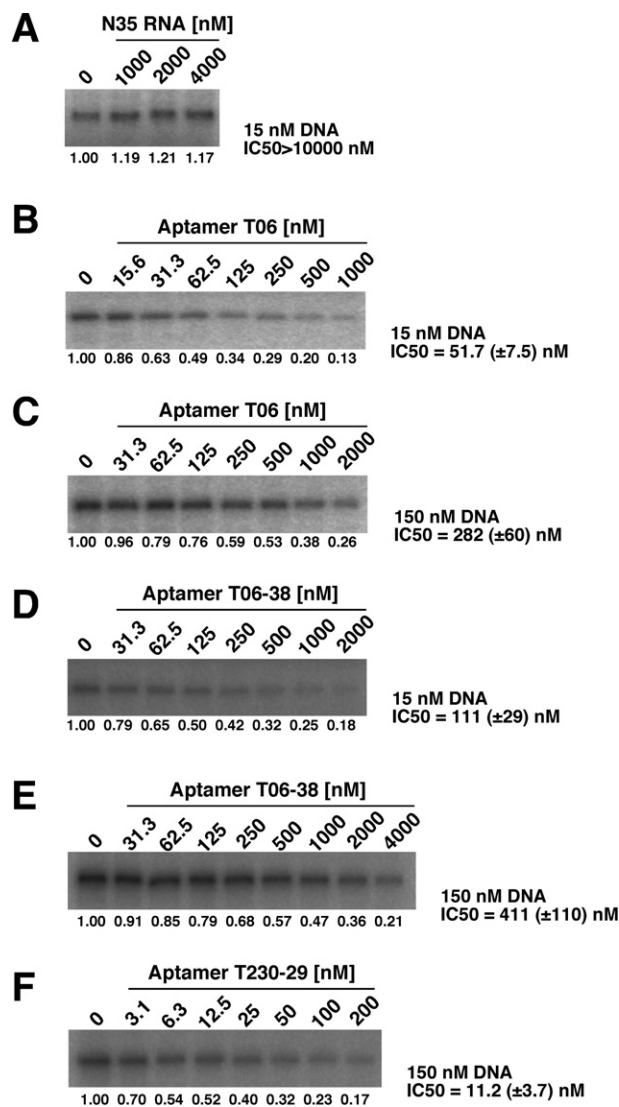


Fig. 2. Estimation of inhibitory activity of the aptamers against T7 RNAP. *In vitro* transcription by T7 RNAP was carried out using the indicated concentrations of template DNA in the presence RNAs. (A) Transcription with 15 nM DNA in the presence of N35 RNA. (B) Transcription with 15 nM DNA in the presence of aptamer T06. (C) Transcription with 150 nM DNA in the presence of aptamer T06. (D) Transcription with 15 nM DNA in the presence of aptamer T06-38. (E) Transcription with 150 nM DNA in the presence of aptamer T06-38. (F) Transcription with 150 nM DNA in the presence of aptamer T230-29. The value under each lane of the gel images indicates the relative amount of transcript compared with that of the same reaction in the absence of RNA. Two independent duplicate experiments were performed, and the mean values are shown.

The dissociation constant (K_D) of aptamer T06 was $8.0 (\pm 2.4)$ nM as estimated by the filter-binding assay (Fig. 1). In contrast, negative control RNAs [the initial pool RNA (N35 RNA) and a 68-nt RNA aptamer (S05 RNA) against SP6 RNAP [21]] showed no apparent binding to T7 RNAP in concentrations of up to 1 μ M (Fig. 1). Under the *in vitro* transcription conditions using a low concentration of template DNA (15 nM), the 50% inhibitory concentration (IC_{50}) value for the aptamer was estimated to be $51.7 (\pm 7.5)$ nM, whereas no inhibitory activity was observed for the negative controls (Fig. 2A, 2B, and Table 2 [21]). The aptamer failed to inhibit SP6 RNAP (31% identical to T7 RNAP) even at concentrations of up to 4 μ M, thus demonstrating its specificity [21]. However, it is noteworthy that higher concentrations of template DNA remarkably weakened the inhibitory activity of the aptamer; in the presence of 150 nM DNA, the IC_{50} value increased to $282 (\pm 60)$ nM (Fig. 2C and Table 2).

Table 1
Primary sequences of RNA aptamers against T7 RNAP.

RNA	Sequence (5' to 3')	Length
T06 ^a	GAGGAUGGCA GCGGAGAGGA AUGGUGUAGU GUAAGUCAAU UCUACUUUCG UUGCAGCCCA CACCACUCUC C	71-nts
T06 - 38 ^b	<u>gUGUAGUGUA AGUCAAUUCU ACUUUCGUUG</u> CAGCCCA C	38-nts
T230 - 29	GCGUAAGUCA AUUCCACUAU CAUUGCUCG C	29-nts

^a The region covering the aptamer T06 - 38 sequence is underlined.

^b The guanosine residue that was not mutagenized in the doped-SELEX pools is indicated by a lowercase letter. Residues completely conserved amongst the first doped-SELEX isolates are indicated in boldface.

Table 2
Inhibitory activities of RNA aptamers against T7 or SP6 RNAPs.

Aptamer	T7 RNAP 15 nM DNA	T7 RNAP 150 nM DNA	SP6 RNAP 50 nM DNA
T06	51.7 (± 7.5) nM	282 (± 60) nM	>10,000 nM
T06 - 38	111 (± 29) nM	411 (± 110) nM	n.a.
T230 - 29	n.a.	11.2 (± 3.7) nM	n.a.
S05	>10,000 nM	n.a.	24.8 (± 6.7) nM

IC₅₀ values for aptamers against T7 or SP6 RNAPs in the presence of the indicated concentrations of template DNA are summarized. The data for aptamer S05 is from a previous report [21].

To elucidate the essential elements of aptamer T06, we examined deletions at the 5'- and 3'-ends and found that 23-nt and 10-nt deletions from 5'- and 3'-terminals, respectively, only slightly affected its activity. The resulting 38-nt variant aptamer T06-38 (Table 1) had a K_D value of 10.5 (± 2.1) nM (Fig. 1). The IC₅₀ values in the presence of 15 nM and 150 nM template DNA were estimated to be 111 (± 29) nM and 411 (± 110) nM, respectively (Fig. 2D, 2E, and Table 2). Further deletions from either 5'- or 3'-terminals drastically reduced the inhibitory activity of the aptamer (data not shown).

3.2. Evolution of aptamer T06-38 by stepwise doped-SELEX

To obtain aptamers with higher inhibitory activity, *in vitro* evolution of aptamer T06-38 was pursued by applying SELEX to an RNA pool comprising 15% mutagenized variants of aptamer T06-38, or by applying doped-SELEX [22]. The inhibitory activity of the pool RNA was progressively increased, with the increase reaching a plateau after 4 rounds (data not shown). Among 74 clones randomly picked out from the fourth round pool, 30 clones showed an activity comparable to that of the parental aptamer (Supplemental Fig. 1). Unfortunately, we failed to find a highly improved aptamer from these clones. However, sequence comparisons revealed apparent sequence conservation among the active clones. In general, both 5'- and 3'-ends were highly tolerant to base replacements, whereas the central regions were less tolerant, and 16 residues were completely conserved (Supplemental Fig. 1 and Table 1).

On the basis of these results, we developed a newly mutagenized RNA pool. For this, 21 residues, not including the 16 residues found to be completely conserved in the first doped-SELEX isolates, were 60% mutagenized and again subjected to doped-SELEX. The inhibitory activity of the pool RNA was comparable with that of the parental aptamer after 2 rounds, and exceeded it after 3 rounds (data not shown). Among the 33 clones in the third round pool, 25 clones showed an inhibitory activity that was at least comparable with the activity of the parental aptamer (Supplemental Fig. 2). According to predictions made using sequence alignment programs based on consensus 2D structure (FoldalignM and MXSCARNA) [23,24], two base-pairing regions were found to be conserved among the inhibitory aptamers (Fig. 3A and Supplemental Fig. 2). These base-pairings were also found conserved among the initial doped-SELEX isolates (Supplemental Fig. 1).

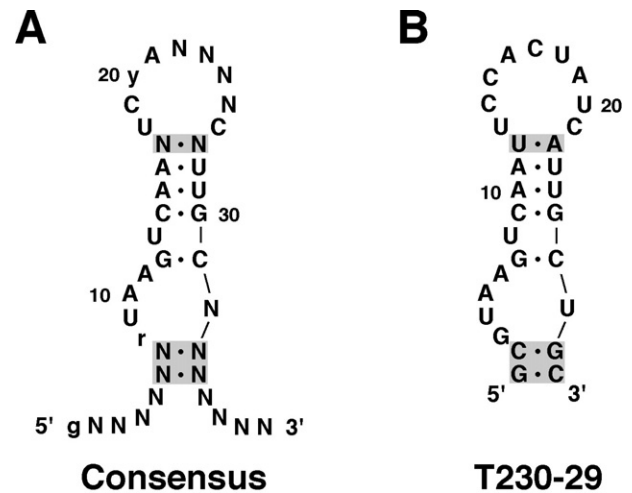


Fig. 3. Predicted 2D structures of the inhibitory aptamers against T7 RNAP. (A) Consensus structures for aptamer T06-38 derivatives predicted by FoldalignM. Completely and moderately conserved sequences are indicated by uppercase and lowercase letters, respectively. Dots denote base-pairings. The conserved base-pairings identified through sequence alignment by FoldalignM and MXSCARNA are highlighted with shadows. (B) 2D structure of aptamer T230-29.

Most isolates had mutations at the 4th, 5th, 35th, and 36th nucleotide positions (24, 22, 24, and 23 clones out of the 25 clones, respectively), suggesting that the residues originally in place at these positions were unfavorable for the activity (discussed below).

Shortened variants, free from the primer-binding sites, were constructed based on the six aptamers with the strongest inhibitory activity (T055, T230, T252, T253, T351, and T851). Among the six variants, only the shortened T230 retained the full inhibitory activity (data not shown), and thus, further truncations of aptamer T230 were examined. Through serial analyses of 5'- and 3'-deletions, the central 29-nt sequence covering the conserved 2D structural motif was identified as necessary and sufficient for full inhibitory activity (Fig. 3B and Table 1). The K_D and IC₅₀ in the presence of 150 nM DNA of this 29-mer variant, T230-29, were estimated to be 2.9 (± 0.9) nM and 11.2 (± 3.7) nM, respectively (Fig. 1, 2F and Table 2).

3.3. Evaluation of the inhibitory mechanism of aptamer T230-29

The transcription inhibition assay described above showed that the template DNA concentrations strongly affect the inhibitory activity of the aptamers (Fig. 2). Therefore, one might expect to find that DNA and aptamers compete with each other for binding to T7 RNAP, with this competition being the basis of the inhibitory mechanism by the aptamers. To confirm this hypothesis, we performed a pull-down experiment using resin-immobilized T7 RNAP (Fig. 4). In the absence of RNA, T7 promoter DNA was found to be associated with the immobilized T7 RNAP, and 15% of the DNA was recovered from the resin (Fig. 4). In contrast, almost no association was observed with

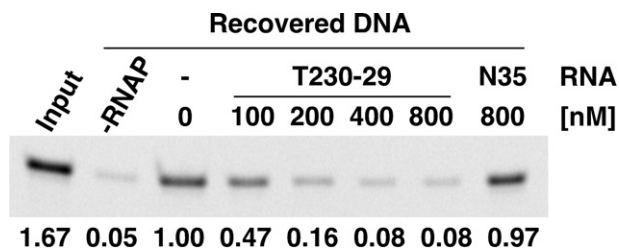


Fig. 4. Evaluation of the inhibitory effect of aptamer T230-29 on T7 RNAP-T7 promoter DNA association. The inhibitory effect of the aptamer on T7 RNAP-T7 promoter DNA association was analyzed by pull-down assay employing resin-immobilized T7 RNAP. Pull-down assays were performed in the presence of the indicated concentrations of RNA. Lanes indicated by “Input” and “-RNAP” show 1/20 volume of the input DNA and the result of the control experiment without the RNAP immobilization. The value under each lane indicates the relative band intensity of the DNA compared with DNA recovered in the absence of RNA. Two independent duplicate experiments were performed, and the mean values are shown.

the control resin without the RNAP immobilization. When aptamer T230-29 was simultaneously added with the DNA, the amount of the recovered DNA reduced in a dose-dependent manner (Fig. 4). In contrast, DNA recovery was rarely affected by the control RNA (N35 RNA). The results supported our hypothesis that DNA and aptamer compete with each other in binding to T7 RNAP.

4. Discussion

In this study, we isolated an inhibitory RNA aptamer against T7 RNAP. By means of an *in vitro* evolution through stepwise doped-SELEX, a 29-nt aptamer with strong inhibitory activity was successfully generated.

The doped-SELEX also revealed sequence/structure requirements for activity (Fig. 3A). Although the conserved motif resided within a central 29-nt region, and although this motif was sufficient for the full activity of one isolate (T230), the original isolate (T06) and most of the examined isolates from the second doped-SELEX required longer sequences in order to be active. One possible explanation for this contradiction is that longer sequences are indispensable for the maintenance of the active structures of these isolates. For example, the expected active structure of aptamer T06-38 is unlikely to be the most stable structure for the sequence (Supplemental Fig. 3) [25–27]. Short deletions from 5'- or 3'-ends are supposed to weaken the active structure (Supplemental Fig. 3A), whilst the inactive structure (Supplemental Fig. 3B) is likely to be less affected. This hypothesis also explains why certain positions were highly replaced among the isolates from the second doped-SELEX. The mutations at these hot spots may stabilize active structures and/or destabilize inactive structures.

In the transcription inhibition assay, template DNA concentration strongly affected the inhibitory activity of the aptamers (Fig. 2), and the pull-down experiment showed that aptamer T230-29 inhibits the association of T7 RNAP to T7 promoter DNA (Fig. 4). Although no similarity was found between the aptamer and the T7 promoter, the aptamer may bind directly to the promoter-recognition residues of T7 RNAP in a manner similar to the promoter DNA. In contrast, the inhibitory activity of the recently identified RNA aptamer against SP6 RNAP [21] is rarely affected by the DNA concentration (unpublished observations), suggesting the involvement of a different inhibitory mechanism in each case.

According to the proposed inhibitory mechanism, the aptamer and T7 RNAP are expected to be of equal stoichiometry. Assuming monophasic concentration dependence for the inhibitory effect, and a Hill coefficient of 1.0 in the transcription assay (Fig. 2), a non-negligible amount of leaky transcription (~10%) was predicted, even in the presence of saturated concentrations of any of the analyzed

aptamers. Alternatively, the data can be interpreted assuming non-monophasic behavior and/or lower values of the Hill coefficient; for instance, if higher aptamer concentrations were to induce autoinactivation, for example, by undesired dimerization [28]. In either case, the utility of these aptamers for efficient transcription regulation is restricted, and further efforts in molecular engineering and/or expression system design are required to overcome these limitations. Previous studies have shown that system design may afford extremely tight control of expression regulation employing repressor proteins [29].

Because T7 and SP6 RNAPs can be employed as platforms for *in vivo* gene expressions [18–20,30–33], aptamers specifically inhibiting these RNAPs would be valuable gene components in the construction of synthetic gene circuits. Several studies have shown that RNA aptamers with affinities similar to (or even lower than) those of the aptamers against the RNAPs can efficiently inhibit their targets' activities *in vivo* [34–38]. In addition, because of their high efficiency and easiness of manipulation, the RNAPs are the most commonly used enzymes for *in vitro* transcription [15–17, 39]. We expect that these orthogonal pairings of RNAPs with the inhibitory aptamers will have applications in the sophisticated design of translation-free, expression regulation systems [40–44].

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.07.004.

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