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Interacting RNA Species Identified by Combinatorial Selection

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Abstract—RNA molecules were selected from a random sequence library for their ability to bind to an RNA stem-loop target. Oligonucleotides with extensive Watson–Crick complementarity to the RNA ligand were selected against by inclusion of a blocking oligodeoxynucleotide in the binding phase of the selection protocol. After 18 generations of SELEX (systematic evolution of ligands by exponential enrichment) a single RNA family was predominant in the binding population. The winning aptamer RNA bound the target RNA with an apparent K_d = 70 nM. Structural mapping and Fe(II)–EDTA protection indicated that the target RNA interacted with small unpaired loops in the aptamer structure. © 1997 Elsevier Science Ltd.

Introduction

RNA–RNA recognition is a feature of many biological processes including translation, post-transcriptional regulation and the action of ribozymes. Most known recognition processes utilize Watson–Crick (W–C) base pairing for specific RNA–RNA recognition. Although W–C interaction is the most common and best-understood mode of nucleic acid interaction, it is not universal. A prominent counter-example is the ribozyme, ribonuclease P, where recognition of the pre-tRNA substrate largely involves shape selection.[1,2] Even where base pairing is used to select RNAs for interaction (e.g., during catalysis by the *Tetrahymena* Group I ribozyme) extensive tertiary interactions within the ribozyme and between ribozyme and substrate affect the strength and specificity of W–C base pairing.[3]

Combinatorial methods offer a powerful rubric for obtaining nucleic acid species capable of binding a variety of molecules. We present here an adaptation of the systematic evolution of ligands by exponential enrichment (SELEX) protocol,[4,5] which we have used to obtain RNA aptamers capable of binding an RNA stem-loop. A blocking deoxynucleotide was used to select against species in the RNA library which bound to the target RNA by W–C base pairing. The winning RNA aptamer was identified by two different selection protocols. Aptamers of this type are analogous to protein antibodies in their ability to recognize the presence of structural domains in complex RNAs (e.g., ribozymes).

*Abbreviations used: W–C, Watson–Crick; nt, nucleotide; SELEX, Systematic Evolution of Ligands by Exponential Enrichment; PCR, polymerase chain reaction.

Data deposition: The sequence of the g18_11 RNA transcript has been deposited in GENBANK, accession number U34759.

Results

Selection of RNAs capable of binding an RNA stem-loop

We used two protocols to identify RNAs that could bind a small stem-loop RNA (Fig. 1a). The buffer conditions used for the binding phase of SELEX were those which support the RNase P reaction, since the recognition of substrate by this ribozyme involves non-W–C interaction.[1]

We wished to avoid the selection of sequences capable of forming extensive W–C base pairs with the ligand. While it might be expected that under the solution conditions employed in these experiments the folded structure of aptamer RNA species would present a substantial activation energy barrier to W–C base pair formation, this could not be ensured a priori. We therefore employed a blocking strategy to remove W–C-complementary molecules from the selectable pool of library RNAs (Fig. 1b). A synthetic oligodeoxynucleotide of the same primary sequence as the RNA stem-loop was mixed in molar excess with the RNA library prior to the binding step of SELEX. This oligodeoxynucleotide would be expected to bind complementary sequences in the library using the same W–C base pairs as would the target stem-loop RNA, thereby removing complementary sequences from the selectable pool of RNAs in the library. On the other hand, since the blocking oligonucleotide lacks the 2′-OH functionality of RNA, it would not be capable of forming the same tertiary interactions with aptamers as would the stem-loop RNA. Therefore, it should not interfere with other, presumably non-W–C, interactions between aptamer and stem-loop RNAs.

Two separate protocols were used to isolate binding aptamers (Fig. 2). In the first (protocol A, Fig. 2a), the stem-loop RNA was oxidized at its 3′-end and

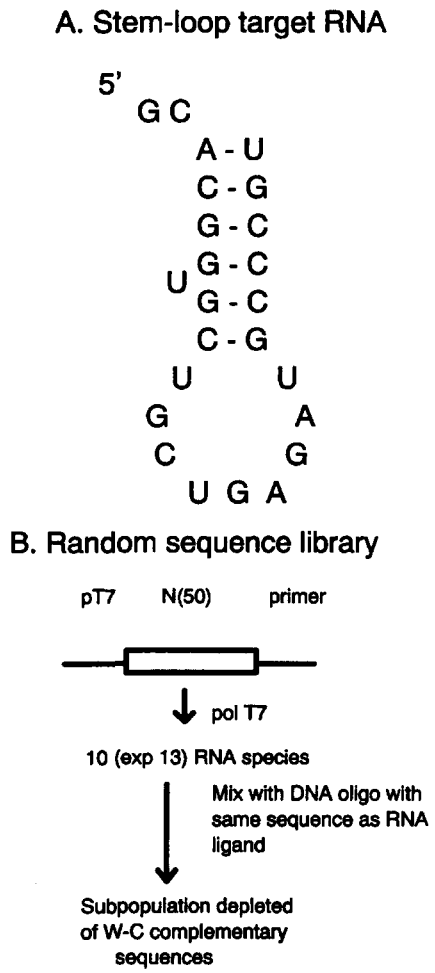

Figure 1. RNA species. (A) The RNA used for selection was a small stem-loop which was prepared by in vitro transcription. (B) Source of selected RNA population. A synthetic library containing 50 randomized nucleotides bounded by constant T7 promoter and 3′-primer sequences was transcribed in vitro. Before each binding step of the selection scheme, the library was mixed with a blocking oligodeoxynucleotide of the same primary sequence as the RNA stem-loop used for selection.

covalently fixed to a Sepharose matrix by hydrazone formation. The RNA library was passed through the affinity column and those species that bound were eluted by reducing the ionic strength and chelating the Mg^{2+} in the solution. In the second protocol (protocol G, Fig. 2b), the RNA library was mixed with radioactively labeled stem-loop RNA before applying the reaction mixture to a nondenaturing polyacrylamide gel. The region of the gel corresponding to slowly migrating complexes was excised (the retarded band was not visible by autoradiography during the early cycles). In either protocol, the selected RNAs were recovered, reverse-transcribed and the cDNA amplified by polymerase chain reaction (PCR). Transcripts of the PCR product were used for the next round of selection.

Sequences of selected RNAs

Both protocols A and G were carried through 12 generations of SELEX; then the DNA sequences of

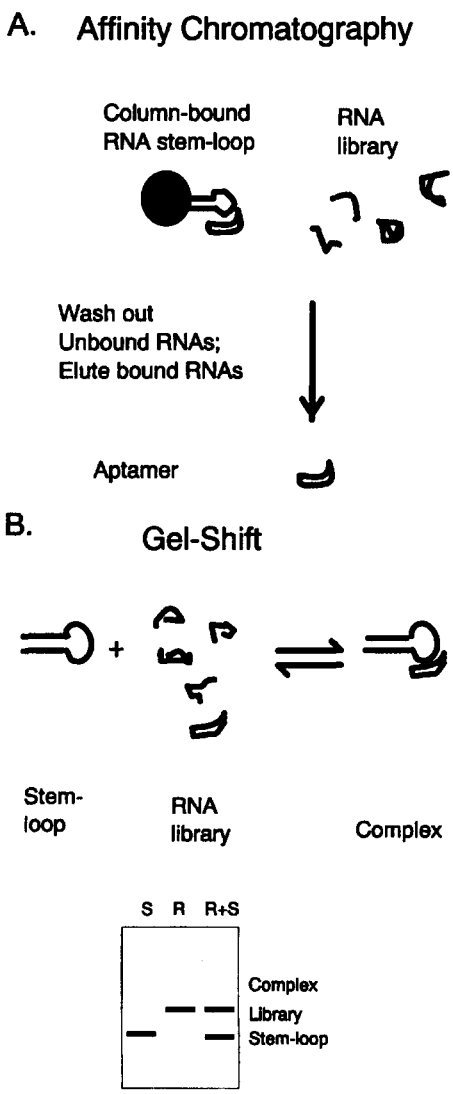

Figure 2. Selection of RNA-binding aptamers. (A) Protocol A: affinity column selection. The stem-loop RNA was oxidized at its 3′-end with $NaIO_4$ and bound to Sepharose adipic acid hydrazide. The population capable of binding the stem-loop RNA was eluted from the column by reducing the ionic strength and $[Mg^{2+}]$. (B) Protocol G: selection of binding RNAs by gel mobility. Radioactively labeled ligand RNA was mixed with the aptamer RNA population and the region migrating more slowly than the stem-loop was excised from the gel. RNAs were eluted, reverse-transcribed and amplified for further rounds of selection.

representative individual clones from each of the populations were determined. Analysis of the sequence complexity of the populations using the PileUp program[6] revealed that sequences derived from protocol A were as related to the sequences derived from protocol G as they were to each other. No single sequence motif was readily apparent in the populations selected through 12 rounds of either protocol. We analyzed the sequence ensemble from protocol G with the Gibbs sampler program of Lawrence et al.[7] Local increases in information per parameter were found corresponding to sequences 3, 6 and 12 long, suggesting the presence of selected motifs of these lengths in the aptamer RNAs.

A.

```
          1                                                  50
g18_01.seq  .......... .......... .......... .......... .t........
g18_04.seq  .......... .......... .......... .......... .....t....
g18_10.seq  .......... .......... t......... .......... ..........
g18_11.seq  .......... .......... .......... .......... ..........
g18_12.seq  .......... .......... t......... .........a ..........
a12_07.seq  .......... .......... .......... .......... ..........
g12_03.seq  .......... .......... .......... ......t... ..........

 Consensus  CCGAAGCATT CCGGCGTAGG GGTCTGTGCG CAAAACCATC GGCCCGGGTG
```

B.

```
          1                                                  50
g18_06.seq  TTCCTTAGGG GTTCAGTTGT TTCGCGCATG AACGGCATCA GTGCACCGTG
g18_09.seq  AGTTGATCGA GCGTGTTAGT TCTCCAATCT CTGTGTAGCG TGGAGCTGGG
```

Figure 3. Sequence analysis of winning RNAs. (A) The most abundant class of cloned sequences is shown. Sequences g18_01, g18_04, g18_10, g18_11, and g18_12 were isolated through 18 rounds of selection by protocol G. Sequence g12_03 was derived through 12 rounds of selection by protocol G, while sequence a12_07 was derived through 12 rounds of affinity column selection (protocol A). (B) Sequences g18_06 and g18_09 were also present in the sequences determined after protocol G through 18 rounds of selection.

A convergent sequence in the selected RNAs

Protocol G was continued for six more generations of SELEX. Individual DNA species in this population were cloned and their sequences determined. Five of the seven sequences determined were closely related (Fig. 3a). A search for these sequences in the set of RNAs sequenced after 12 rounds of SELEX indicated that one clone of this sequence was present in the populations derived by either protocols A or G. A FASTA search of the GenBank database, Release 93, did not identify any statistically significant homologies. We concluded that the sequence family shown in Figure 3a represents a locally optimal solution to the problem of RNA–RNA interaction and that its selection was independent of the protocol used.

The sequences in Figure 3a, with the 5′ and 3′ constant sequences appended, were examined for W–C complementarity with the stem-loop RNA used for SELEX. No continuous complementary sequences greater than four nucleotides (nt) were found. This was concluded to be no more significant than that due to chance (see below). Selection of sequences g18_06 and g18_09 (Fig. 3b), on the other hand, may be the result of W–C complementary interactions between these RNAs and the target RNA sequence used for selection. The sequence of g18_06 contained stretches of six and seven nucleotides, separated by three nucleotides, capable of W–C base-pairing to the RNA stem-loop. Sequence g18_09 contained five contiguous residues complementary to the loop of the target RNA. These RNAs were not examined further.

Strength of selected RNA–RNA interaction

We used a gel-shift assay to estimate the K_d of the selected RNA–RNA interaction (Fig. 4a). A tracer amount of radioactive stem-loop ligand was mixed with various concentrations of g18_04 RNA and the mixtures subjected to electrophoresis. Formation of the RNA–

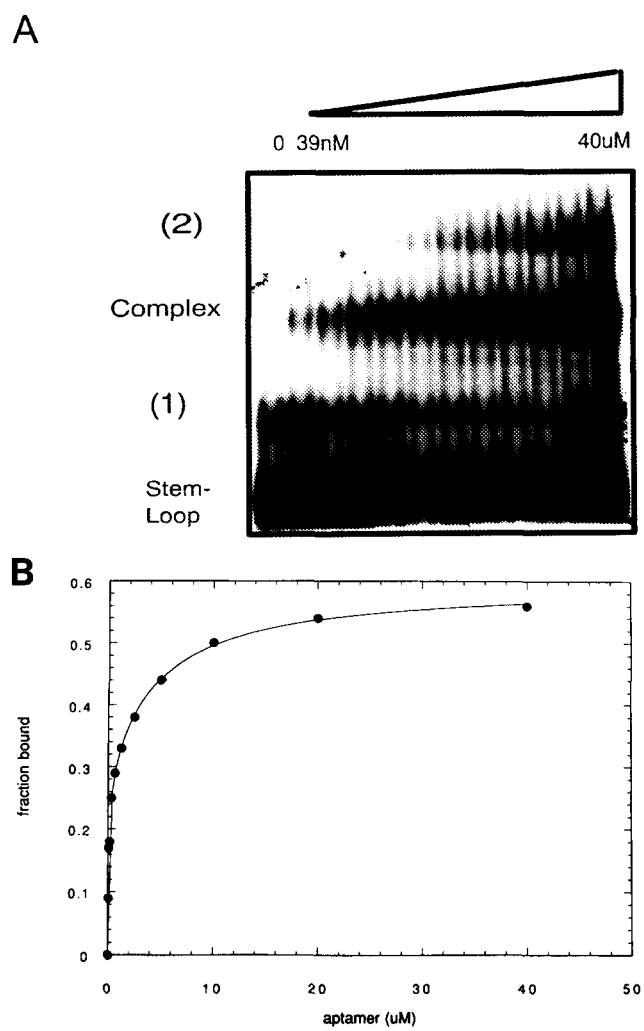

Figure 4. Binding of aptamer g18_04 to radioactive stem loop RNA. A tracer amount of labeled stem-loop was mixed with various concentrations of g18_04 RNA. g18_04-stem-loop complexes were separated by gel electrophoresis and the amount of complex was determined by scanning. (A) Autoradiograph of the gel used for analysis. The band labeled (I) was present in varying amounts in different experiments. It is possible that this species is a dimerized form of the stem-loop which is complexed to the aptamer to form species (2). This apparent event was not included in the analysis of the strength of binding. (B) A binding isotherm showing the amount of binding as a function of g18_04 RNA concentration. The data were fitted to as the sum of two hyperbolae with an overall $R > 0.998$. The first apparent K_d of the interaction was 70 nM.

RNA complex resulted in the electrophoretic retardation of the radioactive ligand. A graphical analysis of the binding data is shown in Figure 4b. The data were fitted to the sum of two hyperbolae. The first fitted K_d was 70 ± 15 nM and the second K_d was 4.4 ± 1.1 μM. The extent of binding was not affected by the presence of blocking oligodeoxynucleotide in molar excess to the aptamer RNA.

Interaction of selected aptamer RNA with RNase P RNA species

The target stem-loop RNA shown in Figure 1 contains the same stem sequence as does the P5.1 domain of *Bacillus subtilis* RNase P RNA, although the loop is

three nt longer than the latter domain. Preliminary experiments (data not shown) indicated that the stem region of the target RNA interacted with the selected aptamer. We therefore examined the ability of the aptamer to be shifted by RNase P RNA variants (Fig. 5). Complex formation could be detected between aptamer g18_01 and *B. subtilis* RNase P RNA. In contrast, the aptamer band was not shifted by incubation with *min1* RNA,[8] a constructed variant of RNase P RNA which lacks several nonconserved domains. We constructed Δ5.1 RNA, a mutant RNase P RNA in which the P5.1 stem-loop (nt 62–81 of the RNase P RNA) was replaced by a *Sna*BI restriction site. Native RNase P RNA bound more strongly to the aptamer than did Δ5.1 RNA, although the latter RNA did interact to some extent with the aptamer. We concluded from this result that Δ5.1 RNA apparently shared aptamer-binding regions with native RNase P RNA and that the selected aptamer did not bind to RNA in general.

Structural studies of the aptamer RNA and its complex with stem-loop ligand

An optimal predicted secondary structure of g18_04 RNA, including the constant sequences 5′ and 3′ to the 50 nt sequence shown in Figure 3a, is shown in Figure 6. This structure was found by the MFOLD program[9] to have a predicted folding energy within 2.0 kcal (8.4 kJ) of the optimal folding for each of the sequences in Figure 3a. The structure was also supported by nuclease digestion studies, the results of which are summarized in Figure 6.

In order to determine potential regions of interaction between the aptamer RNA and the hairpin ligand, we digested complexes between the two molecules with $OH^{\cdot}$ radicals generated by Fe(II)–EDTA and a reductant. This technique[10] detects ribose residues of an RNA which are made inaccessible to the reagent by binding to a molecule. Residues at or near U40 and C60 were protected from digestion by the RNA stem-loop (Fig. 7). Residues G41 through G45 of the aptamer RNA were relatively inaccessible to Fe(II)–EDTA in the absence of ligand, suggesting that they form buried regions of the RNA structure.

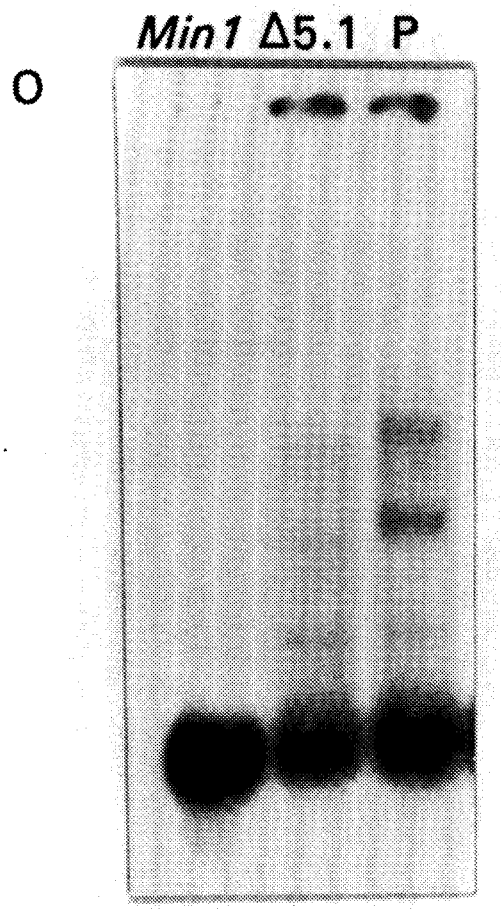

Figure 5. Binding of aptamer g18_01 to RNase P RNA variants. The autoradiogram of a 6% polyacrylamide gel is shown. Radioactive aptamer was mixed with min1 RNA, Δ5.1 RNA, or *B. subtilis* RNase P RNA at 1 μM final concentration and bound species were separated by electrophoresis.

Discussion

Combinatorial selection experiments have been employed to isolate synthetic RNA species capable of recognizing a variety of molecules, including purines, proteins, and metabolic cofactors.[11] In the present work we have extended the scheme of combinatorial RNA selection to the study of intermolecular RNA domain recognition. Previous selections for intermolecular nucleic acid interactions have identified sequences capable of duplex or triplex helix formation. Thus, for example, oligo-A sequences were selected when an RNA library was passed over an oligo-dT column[4] and oligopyrimidine sequences capable of triple helix formation were selected when libraries were selected for the ability to bind a DNA duplex.[12,13] Alternatively, combinatorial selection has been used to define features important for substrate recognition by RNase P.[2]

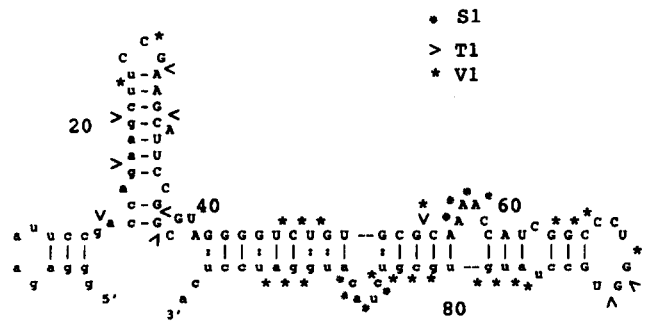

Figure 6. Predicted secondary structure of aptamer g18_04. Primer sequences at the 3′ and 5′ ends of the transcript are indicated in lower case and the unique portion of the molecule is indicated in capital letters. Nuclease digestion data, summarized in the figure are consistent with this structure and further constrain the folding algorithm in favor of the structure shown. No alternative structures were predicted by the MFOLD program if these constraints were included in the algorithm. Digestion of the loops near nt 79 and 25 may reflect pseudoknot formation between these loops, although there are no covariation data to support this possibility.

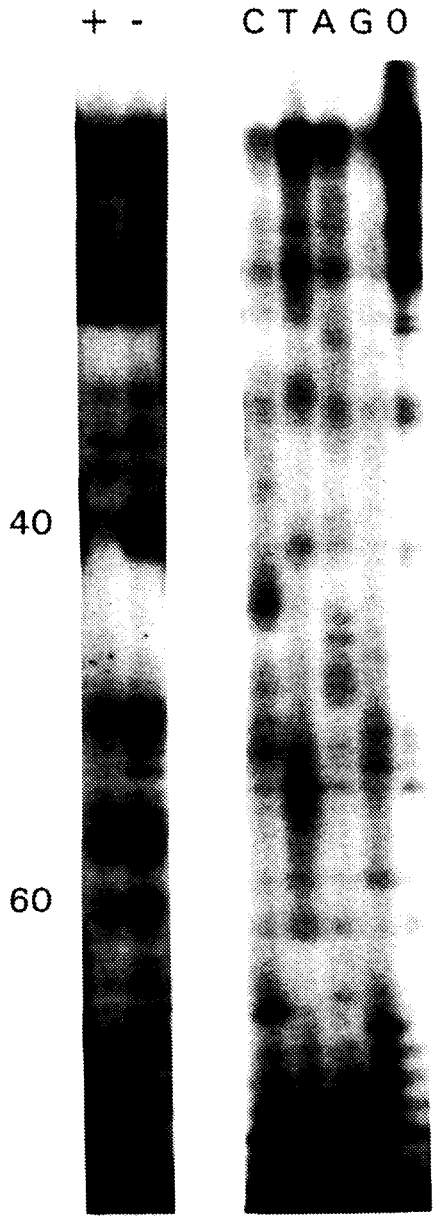

Figure 7. Chemical protection analysis. Aptamer RNA was reacted with Fe(II)–EDTA in the presence (lane (+)) or absence (lane (−)) of stem-loop RNA. The RNA was reverse-transcribed to identify regions of diminished chain breakage. A sequencing ladder (lanes G, A, T, and C) and the results of reverse transcription of unmodified aptamer (lane 0) are shown for comparison.

In order to obtain RNA species capable of recognizing the stem-loop RNA domain in the absence of extensive W–C bp complementarity, we employed a counter-selection strategy, adding a molar excess of a DNA oligomer of the same sequence as the RNA ligand in the selection phase of the protocol. We reasoned that the presence of this DNA sequence would bind those species in the RNA library which were capable of extensive base-pair formation with the RNA ligand, thereby rendering these sequences incapable of being identified by the selection scheme.

The efficacy of this stratagem was borne out by analysis of the sequences of the selected RNAs. A single predominant sequence was identified after 18 generations of SELEX. We consider it highly significant that this winning sequence was found in earlier populations (12 generations) selected either by affinity chromatography or gel-shift experiments. This observation argues against the winning sequence being an artifact of the selection process, for example, a matrix-binding aptamer. The presence of the winning RNAs in the twelfth-generation populations (two out of 100 sequences) also allows an estimation of the strength of the selection schemes by solving the expression $x^{12} = 0.02/10^{13}$. This analysis indicates that each round of selection enriched the aptamer population approximately 8.7-fold for the strongest binding species, an enrichment per generation close to the 10-fold noted in other applications of SELEX.[11]

Although unrelated sequences can be written as complementary if enough gaps are allowed,[14] several observations support the contention that interaction between the aptamer and stem-loop RNAs is not determined solely by W–C interaction. First, the binding of the winning aptamer to the target RNA stem-loop was unaffected by a molar excess of deoxynucleotide with the same primary sequence as the stem-loop (B.C. and F.J.S., unpublished experiments). By contrast, if W–C interactions were responsible for the interactions observed in Figure 6, preincubation with the deoxynucleotide sequence would be expected to decrease the extent of RNA–RNA binding. Secondly, the extent of complementarity between aptamer and stem-loop RNAs was only 4 nt of contiguous sequence, well within the extent predicted to occur between two random sequences 24 and 50 nt long. Consider that the 50-mer sequence imbedded in the selected RNA represents 20% of all possible tetrameric sequences. Then the probability of not finding a given tetramer is 0.80. There are 18 different tetrameric subsequences in the 24-nt ligand RNA sequence. Therefore, the probability of not finding any matches of length 4 between the two sequences is $(0.80)^{18} = 0.02$ (i.e., there is a 98% probability of this degree of complementarity occurring by chance). This probability rapidly increases as the region of complementarity searched for becomes longer so that, for example, the probability of finding a 6-nt match in two random sequences of this size is 0.23. Again suggesting that the interaction between the two molecules is not solely due to W–C interaction, the short regions of W–C complementarity between the winning aptamer and stem-loop RNA include portions of both the stem and loop of the latter RNA. Since the loop region of the target RNA would be expected to be most available for W–C base pairing in solution, this further argues that W–C bp formation is not the sole mode of interaction between the molecules. Finally, the chemical protection experiment shown in Figure 7 indicated that the interaction of the target RNA with the selected aptamer involved backbone or minor-groove interaction[10] with looped residues at or near nucleotides 40 and 60 of the aptamer molecule. Other subsequences complementary to the stem-loop motif (e.g., nt 42–45) within the

aptamer were not solvent-accessible as indicated in Figures 6 and 7.

Incubation of radiolabeled aptamer with *B. subtilis* RNase P RNA resulted in a retarded mobility of the aptamer in a nondenaturing polyacrylamide gel. In contrast, the mobility of the aptamer was not reduced by incubating it with a constructed RNase P RNA, *min1* RNA, which lacks several nonconserved domains of RNase P RNA.[8] Therefore, the aptamer is not a nonspecific RNA-binding molecule. Some retardation occurred between Δ5.1 RNA and the aptamer, although this was quantitatively weaker than the interaction between native RNase P RNA and the aptamer. It is possible that one or more features of the P5.1 domain (e.g., a bulged stem) are present on another domain of Δ5.1 RNA and are responsible for binding of the aptamer. Whatever the precise mechanism of interaction, the data in Figure 6 indicate that the aptamer is capable of differentiating closely related RNAs.

Conclusions

The winning aptamers described here are analogous to a conventional or monoclonal antibody in their ability to distinguish the presence of a domain in a larger molecule. They could be valuable in studies of the architecture or assembly of larger (e.g., catalytic) RNAs. The methods of combinatorial selection and counterselection described here should allow RNA aptamers capable of specific RNA recognition to be generated with relative ease.

Experimental

Synthetic RNA library

The library used, a gift of Professor A. D. Ellington (Indiana University), contained 50 randomized nucleotides bounded by the promoter sequences AGTAATACGACTCACTATAGGGAGAATTCCGACCAGAAG and TGAGGATCCATGTAGACGCACATA at the 5′ and 3′ regions, respectively. The library was amplified through five cycles of PCR. One microgram (approximately 10^{13} species; see ref 4) of this population was transcribed with T7 RNA polymerase (Epicentre Technologies, Madison, WI) in a 20-μL reaction according to the manufacturers directions. Transcripts were purified by gel electrophoresis through denaturing polyacrylamide gels. The purified RNA library (30 μg) in 0.1 mL 10 mM TrisCl, pH 8.0/100 mM NaCl was brought to 70 °C for 10 min and slowly cooled to room temperature at which time the solution was made 25 mM in $MgCl_2$. The blocking oligonucleotide (GCACGGTGCTGCTGAGATGCCCGT, 2.6 μg) was added and the mixture kept at room temperature for 1 h before selection.

Affinity column chromatography

The RNA stem-loop was synthesized by transcription from the template ACGGGCATCTCAGCACCGTGCTATAGTGAGTCGTATTAC to which the T7 promoter oligonucleotide AGTAATACGACTCACTATA had been annealed.[15] The resulting RNA stem-loop (sequence GCACGGUGCUGCUGAGAUGCCCGU), 145 μg, was dissolved in 100 μL of 0.1 M K-phosphate, pH 8.0. Then 50 μL of freshly prepared, ice-cold 20 mM $NaIO_4$ were added and the solution kept on ice in the dark for 2 h. The RNA was recovered by ethanol precipitation and redissolved in 0.1 mL of 0.1 M K-acetate, pH 5.0. The oxidized RNA was coupled to 0.5 mL of packed Sepharose–adipic acid hydrazide resin (Pharmacia) at 4 °C overnight, with gentle mixing. Efficiency of coupling was determined to be >95%. The RNA library was preselected through a column (0.5–1 mL bed volume) of Sepharose–adipic acid hydrazide in P buffer (10 mM TrisCl, pH 8.0/100 mM NaCl/25 mM $MgCl_2$) and then applied to a column (0.5 mL bed volume) of RNA-coupled resin. The affinity column was washed with 5 mL of P buffer and then the bound RNA population was eluted with two column volumes of 25 mM Na–EDTA, pH 8.0. The RNA was recovered by ethanol precipitation using 200 μg glycogen as carrier, reverse-transcribed (Superscript I, Bethesda Research Laboratories) and amplified by 10 cycles of PCR.

Selection of aptamer RNAs by gel mobility shift

The stem-loop RNA was uniformly labeled with [α-^{32}P] CTP during transcription. Library RNAs, derived by transcription, were purified by electrophoresis in a denaturing polyacrylamide gel. Library RNAs were preincubated in B buffer (10 mM TrisCl, pH 8.3/25 mM $MgCl_2$/100 mM NaCl/165 mM KCl) at 70 °C for 10 min in the presence of a fixed concentration of labeled target RNA and cooled to room temperature. The samples were loaded on a 6% or 8% polyacrylamide gel in E buffer (100 mM Tris–Hepes, pH 8.3/1 mM $MgCl_2$/1mM KCl/0.1 mM EDTA). Complexed RNA species which migrated more slowly than free stem-loop RNAs were eluted from the gel by electrophoresis into a dialysis bag and amplified as above.

DNA manipulations

Eluted RNAs were reverse-transcribed to generate complementary DNAs, which were then amplified by PCR. Amplified DNA fragments were digested with EcoR1 and BamH1 and ligated into pGEM-3Z(+) (Promega Biotech). Transformation was into INVαF′ competent cells (Invitrogen) or DH5α. Sequences of the inserted sequences were determined by the chain-termination method using Sequenase version 2.0 (United States Biochemical). Sequences of the inserted sequences were determined by the chain-termination method using Sequenase version 2.0 (United States Biochemical). Mutant Δ5.1 of *B. subtilis* RNase P RNA,

which contains a recognition sequence for *Sna*BI in place of the P5.1 element, was constructed by the procedure of Stemmer and Morris[16] using the oligonucleotides AGATCGTCTCTACGTAGTGTTCGTGCCTAGCGAAGT and AGAACGTCTCTACGTAGCGAGCATGGACTTTCCTCT. The inverse PCR product was digested with *Bsm*BI endonuclease and religated. The identity of the RNase P construct was confirmed by digestion with *Sna*BI and by DNA sequencing.

Binding of cognate RNAs

RNA–RNA binding constants were estimated by electrophoretic mobility shift assay. The inserted sequences of plasmid constructs including the T7 promoter region were amplified by PCR and transcribed using T7 RNA polymerase. Stem-loop RNA was uniformly labeled by transcription with [α-^{32}P]CTP. Aptamer RNAs at different concentrations were heated in B buffer (10 mM TrisCl, pH 8.3/25 mM $MgCl_2$/100 mM NaCl/165 mM KCl) at 70 °C for 10 min either in the presence of or before mixing with a fixed concentration of labeled stem-loop RNA and slowly cooled to 4 °C. The samples were applied to a 6% polyacrylamide or 3% agarose gel which had been prerun in E buffer. Electrophoresis was in E buffer at constant voltage of 80–100 V; temperature was maintained at 4 °C. Quantitation of free and complexed stem-loop RNA was by autoradiography and Beta scanning.

Binding experiments to distinguish among RNase P RNA species were performed by mixing radiolabeled aptamer RNA with 0.8 μM RNase P RNA in 10 mM Tris–HCl, pH 8.3/12.5 mM NaCl/12.5 mM KCl/15 mM $MgCl_2$. The reaction mixture was kept at 70 °C for 5–10 min, followed by incubations at room temperature for 40 min and at 4 °C for >2 h. The samples were applied to a 6% polyacrylamide gel as above.

Fe(II)–EDTA cleavage

The reaction mixture contained 0.3 μM aptamer RNA, with or without 14 μM stem-loop RNA, in 100 μL B buffer. The solution was heated at 80 °C for 1 min and cooled slowly to room temperature. The reaction was initiated by centrifuging into the solution 1 μL of each of the following freshly prepared reagents: 0.4 mM $Fe(NH_4)_2SO_4 \cdot 6H_2O$, 0.8 mM EDTA, pH 8, 0.6% H_2O_2, and 50 mM dithiothreitol. The reaction was terminated after 1 min by addition of thiourea to a final concentration of 1.3 mM. RNA was recovered by ethanol precipitation and reverse-transcribed using the same primer as was used in library amplification.

DNA sequence analysis

DNA sequence analysis, including database searching and structure prediction, was done with the GCG software package (Genetics Computer Group, Madison, WI). The Gibbs sampler program of Lawrence et al.[9] was implemented at the Pittsburgh Supercomputing Center, as were the programs used to analyze the Gibbs sampler output (H.B.N. and F.J.S., manuscript in preparation).

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