METHOD

Streptavidin aptamers: Affinity tags for the study of RNAs and ribonucleoproteins

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ABSTRACT

RNA affinity tags would be very useful for the study of RNAs and ribonucleoproteins (RNPs) as a means for rapid detection, immobilization, and purification. To develop a new affinity tag, streptavidin-binding RNA ligands, termed “aptamers,” were identified from a random RNA library using in vitro selection. Individual aptamers were classified into two groups based on common sequences, and representative members of the groups had sufficiently low dissociation constants to suggest they would be useful affinity tools. Binding of the aptamers to streptavidin was blocked by presaturation of the streptavidin with biotin, and biotin could be used to dissociate RNA/streptavidin complexes. To investigate the practicality of using the aptamer as an affinity tag, one of the higher affinity aptamers was inserted into *RPR1* RNA, the large RNA subunit of RNase P. The aptamer-tagged RNase P could be specifically isolated using commercially available streptavidin-agarose and recovered in a catalytically active form when biotin was used as an eluting agent under mild conditions. The aptamer tag was also used to demonstrate that RNase P exists in a monomeric form, and is not tightly associated with RNase MRP, a closely related ribonucleoprotein enzyme. These results show that the streptavidin aptamers are potentially powerful tools for the study of RNAs or RNPs.

Keywords: affinity chromatography; aptamer; biotin; in vitro evolution; ribonucleoprotein; streptavidin

INTRODUCTION

Affinity tags for protein isolation and detection have proven very useful for a wide variety of research applications; examples include FLAG epitope (Prickett et al., 1989), glutathione S-transferase (Simons & Vander Jagt, 1981), polyhistidine (Porath et al., 1975; Porath, 1992), or protein-A tags (Stahl & Nygren, 1997). The study of RNAs or ribonucleoproteins (RNPs) could also be facilitated by a highly specific RNA affinity tag that allows a selective recovery of the tagged RNA and all associated proteins under nondenaturing conditions. A limited number of RNA affinity tags have already been identified (Bachler et al., 1999; Srisawat & Engelke, 2001), but these tags have limitations in terms of recovering native RNP complexes in the absence of artificially bound macromolecules. An alternative approach to the problem is to develop an RNA tag that binds tightly to a commonly

available target molecule in such a way that the ligand–RNP complex can be selectively and gently dissociated afterwards.

To satisfy these requirements, we have developed RNA tags that bind specifically to streptavidin and can be gently eluted by competition with biotin. Streptavidin is a homotetrameric protein produced by *Streptomyces avidinii*. Each mole of streptavidin can bind to four moles of biotin with extraordinary high affinity, making streptavidin–biotin affinity a favorite tool for many applications in molecular biology (reviewed in Wilchek & Bayer, 1990a, 1990b; Diamandis & Christopoulos, 1991). The dissociation constant (K_d) for the biotin interaction is approximately 10^{-14} M (Green, 1990), one of the strongest noncovalent biological interaction known in nature. Streptavidin was chosen as a target of the RNA affinity tag because it is commercially and easily available in various forms, either as a purified protein or as conjugated forms with enzymes, fluorescent dyes, or supporting matrices. This would make a streptavidin-specific RNA affinity tag particularly useful for later applications such as detection or purification.

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RNA ligands for streptavidin were isolated from a large pool of random-sequence RNA molecules through in vitro selection, or SELEX (systematic evolution of ligands by exponential enrichment; Ellington & Szostak, 1990; Tuerk & Gold, 1990). These RNA "aptamers" could be specifically eluted from streptavidin with biotin, and we further tested the practicality of using this aptamer as an RNA affinity tag to isolate rare ribonucleoprotein complexes. The RNA subunit of *Saccharomyces cerevisiae* nuclear RNase P was tagged with a streptavidin-binding aptamer in a nonessential stem/loop that extends out from the enzyme into solution. This aptamer insertion was shown not to have adverse effects on holoenzyme assembly or function, and was used to explore the form of native ribonucleoprotein complexes containing RNase P. The RNase P isolation using streptavidin-agarose beads was found to be efficient and specific to the aptamer tag. In addition, elution with biotin that was done under mild conditions resulted in recovering RNase P with its activity still preserved. The study showed the feasibility of using the streptavidin aptamer as an RNA affinity tag for RNA affinity purification, as well as its potential as a tool in molecular biology.

RESULTS

In vitro selection and characterization of streptavidin-binding aptamers

A library of 84-nt RNAs was generated in which the 40-nt sequence in the center is variable and flanked by fixed sequences on each end. The complexity of the library was estimated to be greater than 10^{16} sequences. RNA aptamers that specifically bind to streptavidin were initially isolated from the RNA library by binding to streptavidin conjugated to agarose beads for six rounds of selection and amplification. In each round, streptavidin-agarose beads were incubated with the RNA pool in a binding buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 100 mM NaCl), washed with binding buffer, and eluted with an elution buffer (8 M urea and 5 mM EDTA). RNA was amplified by reverse transcription, polymerase chain reaction, and T7 RNA polymerase transcription to provide the RNA pool for the next round of selection. The aptamers were further selected for binding to purified streptavidin for another three rounds using electrophoretic mobility shift, after which individual selected sequences were cloned into plasmids. The RNAs produced from 17 aptamer clones were tested for their ability to bind to streptavidin by electrophoretic mobility shift assays. Eight of the RNA aptamers bound well to streptavidin in these assays and their sequences were determined. The aptamers were classified into two groups based on the consensus sequences (Fig. 1A). Most of them (six out of eight) belong to group 1. To

A

Group 1

S1 UCAUGCAAGUGCGUAAGAUAUCGCGGGCGGGGGCGUAAU
 S2 UCAGUUAAAGUGUGUAAGAUAGCACGGGUCGGGAUGGCGG
 S8 UCAUGGAAGUCCCUAAGAUAUGGGGGGUCGGGGAGACAA

Group 2

S12 GGAGUAGAUGUUCUGCUGCGGCAAGGAGCA CGUGGCGUUU
 S13 GAGUCUUCGAGUGCGGCAAGGAGCA UUUAGGGGUAGAUGU

FIGURE 1. Streptavidin-aptamer sequences. **A:** Classification of streptavidin aptamers. The aptamers are classified into two groups based on the consensus sequences (underlined). Only the randomized regions of the aptamers (position 20 to position 59) are shown and aligned. Six out of eight aptamers belong to group 1 (S1 was found to appear four times during the screening). **B:** The predicted secondary structures of group 1 aptamers. The sequence from position 8 to position 52 of each aptamer from group 1 is predicted to form conserved secondary structures. The conserved structures are 45 nt long encompassing the 5' constant region and the conserved sequences. The predicted structure of full-length S1 is also shown for comparison.

determine the dissociation constants of the aptamers to streptavidin, S1 and S13 aptamers were chosen as the representatives from group 1 and 2, respectively. S1 aptamer bound to streptavidin with higher affinity than S13, with apparent dissociation constants of approximately 7.0 and 20×10^{-8} M, respectively.

The structures of the aptamers were analyzed using a secondary-structure prediction algorithm (Mathews et al., 1999) and by covariation analysis. The group 2 aptamers did not produce any noticeably reproducible structure, whereas those from group 1 share the predicted structure shown in Figure 1B. The structure is 45 nt long, spanning from position 8 to position 52. This contains part of the constant sequence of the 5' flanking region and all of the indicated conserved nucleotides in the randomized region. The truncated form of S1 containing this minimal sequence from the above region was shown to bind as efficiently to streptavidin as the full-length S1 (data not shown), suggesting that this conserved structure might form a minimal binding motif shared by all group 1 aptamers.

The binding of aptamers to streptavidin was blocked and dissociated by biotin

The effect of biotin on the aptamer binding to streptavidin was investigated. Binding of aptamers to streptavidin is blocked if the streptavidin is first incubated with sufficient biotin to saturate all of its biotin-binding sites (Fig. 2). In addition, when the preformed aptamer/streptavidin complexes were incubated with biotin, the aptamer binding diminishes over time in a concentration-dependent manner (Fig. 3). S13 appeared to elute much more quickly in the presence of biotin. The aptamer could be released completely and rapidly at the concentration of biotin that is equimolar or higher than biotin-binding sites of streptavidin (Fig. 3B, lower panel, transition from 0.5–1.0 μ M biotin). In contrast, S1 aptamer was released more slowly, even at the relatively high concentrations of biotin. Explanations for the interference effect of biotin might be as the following: (1) the aptamer-binding sites might be near or at the biotin-binding pocket of streptavidin, making them compete with each other to bind to streptavidin; or (2) the binding of biotin might cause a conformational change on streptavidin, thus making the aptamers unable to recognize and bind to streptavidin. Although the mechanisms of biotin interference are not yet clear, this property makes possible the use of biotin as a highly selective agent to elute the aptamer-tagged RNAs from streptavidin. Biotin elution can be accomplished under mild conditions by simply adding biotin into the binding buffer. Therefore, the structures and functions of the eluted RNAs or RNPs are likely to remain intact. In contrast, commonly used elution techniques for most affinity tags require harsh or denaturing conditions such as using lower pH, denaturing agents, or removal of divalent cations. This occasionally results in irrevers-

FIGURE 2. The binding of aptamers to streptavidin is blocked by prebinding biotin. Radiolabeled S1 or S13 aptamers were incubated with either free streptavidin or streptavidin previously blocked with biotin. Free RNA was then separated from RNA/streptavidin complexes by electrophoresis through polyacrylamide gels under non-denaturing conditions. It is not known whether the second electrophoretic form of free S13 RNA is dimeric or some other folding isoform.

FIGURE 3. Dissociation of aptamers from RNA/streptavidin complexes by biotin over time in a concentration-dependent manner. Radiolabeled S1 or S13 aptamers (20 nM) were incubated with 250 nM streptavidin to generate preformed RNA/streptavidin complexes. The complexes were then either incubated with 2 mM biotin for different incubation periods (A), or with various concentrations of biotin for 2 h (B). The samples were then analyzed by electrophoretic mobility shift assays. The concentration of tetrameric streptavidin in the assay is approximately 250 nM, providing approximately 1,000 nM (maximum) binding sites for biotin. Note that at the concentration of 1,000 nM biotin, S13 aptamer is abruptly eluted.

ible losses of the organization, structures, and functions of the target molecules.

Using streptavidin aptamers as RNA affinity tags

To evaluate the feasibility of using streptavidin aptamers as affinity tags in RNP complexes, the RNA subunit of *S. cerevisiae* nuclear RNase P was chosen as a model target to be tagged with streptavidin aptamers. RNase P is an endoribonuclease that cleaves the 5' end of pre-tRNA to generate a mature 5' end of tRNA. Nuclear RNase P has one 369-nt RNA subunit, *RPR1* RNA (Lee et al., 1991), and at least nine integral protein subunits. The full-length S1 aptamer was used as an affinity tag for the RNA subunit. The aptamer was incorporated between the base positions +133 and +140 of the wild-type *RPR1* RNA. This region of *RPR1* RNA has been shown to be exposed to the solvent and not essential for function, making it a good candidate site to be tagged with the aptamers (Pagán-Ramos et al., 1994, 1996; Tranguch et al., 1994; Fig. 4A).

The S1-tagged *RPR1* gene was placed in a low-copy, *LEU2*-marked yeast plasmid and transformed into a yeast strain in which the chromosomal copy of *RPR1* had been disrupted. Because *RPR1* is an essential gene, the strain originally also contained a wild-type *RPR1* gene on a low-copy plasmid containing a *URA3*

FIGURE 4. Affinity tagging of *RPR1* RNA, the RNA subunit of nuclear RNase P with the S1 streptavidin aptamer. **A:** S1 aptamer was inserted into the gene for *RPR1* RNA between positions +133 and +140 in the original RNA sequence, and the RNA was expressed in yeasts. *NdeI* restriction sites flanking the aptamer were also created to facilitate future removal or insertion of alternative RNA affinity tags into *RPR1*. **B:** The yeast strain with the wild-type *RPR1* gene deleted, but expressing S1-tagged *RPR1* (S1-*RPR1*), has a growth phenotype comparable to that of the wild-type strain. **C:** The northern blot analysis shows no detectable defect in maturation of S1-tagged *RPR1* RNA. *RPR1* RNA is processed from a precursor to a mature form. A similar pattern is observed with S1-tagged *RPR1* RNA, except that both forms are longer due to the aptamer tag.

marker. After the S1 aptamer-tagged *RPR1* gene was transformed into the strain, loss of the wild-type gene could be selected by growth on 5-fluoroorotic acid. It was found that the aptamer-tagged *RPR1* RNA compensated for the loss of the essential, wild-type *RPR1* RNA. The yeast cells expressing only the aptamer-tagged *RPR1* had a growth phenotype indistinguishable from the strain expressing the wild type (Fig. 4B). Moreover, northern blot analysis of *RPR1* RNA showed that the expression of S1-tagged *RPR1* RNA was similar to the patterns for normal *RPR1* RNA, with a small amount of pre-*RPR1* transcript and a larger amount of the smaller, mature form (Lee et al., 1991). This suggests that the tagged *RPR1* RNA is assembled into holoenzyme and processed normally (Fig. 4B), as previous studies have shown that failure to properly assemble holoenzyme leads to more precursor and less mature *RPR1* RNA (Ziehler et al., 2001).

Isolation of RNase P using the aptamer can preserve the activity

Preliminary data showed that RNase P containing S1-tagged *RPR1* RNA could be isolated with streptavidin-agarose more efficiently than S13-tagged *RPR1* RNA (data not shown), possibly due to the higher affinity of S1 for streptavidin. Therefore, only the S1 affinity tag was used in subsequent studies. Soluble extracts from yeast strains expressing either wild-type or S1-tagged *RPR1* RNA were prepared, and RNase P was isolated using streptavidin-agarose. One potential problem in the purification is that yeasts contain a substantial

amount of free biotin and biotinylated proteins that could block the binding of the aptamer tag to streptavidin beads. Physical removal of the biotin and biotinylated proteins from the extracts was not practical, but it was found avidin from egg white could be used to absorb biotin in yeast extracts. Egg-white avidin (Green, 1990) binds very tightly to biotin, with an affinity comparable to that of streptavidin, but the S1 aptamer shows no significant tendency to bind efficiently to egg-white avidin (see Materials and Methods). Therefore, addition of avidin, which is reasonably inexpensive and commercially available, was a convenient method to render harmless the free biotin and biotinylated proteins in yeast extracts. This was done by incubating 5 μ g of avidin to every milligram of protein in yeast extract and incubating for 10 min at 4°C before the extracts were incubated with streptavidin-agarose.

After the binding step, the beads were extensively washed and eluted with 5 mM biotin in binding buffer. The eluates were analyzed for the presence of *RPR1* RNA as well as the activity of RNase P.

In Figure 5A, the northern blot analysis shows that only the *RPR1* RNA (and pre-*RPR1* RNA) containing the aptamer tag is specifically isolated with streptavidin. Approximately 20–25% of the input *RPR1* RNA is recovered in the eluate; a reasonable yield considering that the isolation was carried out directly from crude extracts, where nuclear RNase P is present at very low abundance (less than 400 molecules/cell; Pagán-Ramos et al., 1996). In crude extracts, we expect severe nonspecific competition to mask the exposed aptamer RNA (e.g., by nonspecific RNA binding pro-

FIGURE 5. Isolation of S1-tagged RNase P. Soluble extracts either from wild-type or S1-RPR1 strains were incubated with streptavidin-agarose, washed, and gently eluted with biotin in the continued presence of binding buffer. **A:** The presence of *RPR1* RNA in the input and the eluate fractions was analyzed by northern blot analysis. Only RNase P containing the aptamer-tagged *RPR1* RNA from S1-RPR1 strain can be isolated using streptavidin-agarose. **B:** RNase P in the input and eluate are assayed for RNase P activity. The enzyme cleaves the substrate pre-tRNA to give mature tRNA and the 5' leader. Input fractions of crude extract consistently have high levels of nucleases that degrade the radiolabeled substrate and products, with the 5' leader product being particularly susceptible.

teins) and to the streptavidin. The RNase P that could be bound was efficiently eluted with biotin and was found to be catalytically active, at approximately the expected levels for the recovery predicted by the northern analysis (Fig. 5B). (Quantitation of RNase P activity in crude extracts is difficult due to the presence of inhibitors and other nucleases.) As expected, the eluted RNase P fraction was less contaminated than the crude extract with other nucleases. Protein content in the eluate was about 5,000–10,000-fold lower than that in the input, resulting in a purification of at least 2,500-fold in a single purification step.

Studying the organization of RNase P using the aptamer tag

Nuclear RNase P of *S. cerevisiae* consists of at least nine known proteins subunits, and eight out of the nine

protein subunits are also subunits of a related ribonucleoprotein enzyme, RNase MRP. This raises a question of whether RNase P may associate with RNase MRP or other RNase P holoenzymes in a large ribonucleoprotein complex through various subunit interactions. To study the organization of RNase P, a yeast strain expressing both wild-type and S1-tagged *RPR1* was generated. Soluble extracts were then incubated with streptavidin-agarose, washed in binding buffer and eluted with biotin. If RNase P exists as a dimer, the existence of both wild-type and S1-tagged *RPR1* RNAs is expected to produce a significant percentage of heterodimers containing both RNAs. Similarly, if a significant amount of RNase P is part of a large complex also containing RNase MRP, the RNase MRP RNA subunit (*NME1* RNA) should be coprecipitated with the aptamer-tagged RNase P and should be detected in the eluate. Because the binding and elution steps were done under mild conditions, it should be possible to detect even a weak association. However, as shown in Figure 6, neither *NME1* RNA nor wild-type *RPR1* RNAs are present in the eluate, suggesting that RNase P holoenzymes are likely to exist in monomeric forms and not closely associated with RNase MRP. This result is consistent with previous studies that showed RNase P and RNase MRP could be chromatographically separated,

FIGURE 6. Testing whether RNase P is present as dimers or in complexes with RNase MRP. A yeast strain expressing both wild-type and S1-tagged *RPR1* RNA was generated. Tagged RNase P was then isolated using streptavidin-agarose from soluble extracts and analyzed for *RPR1* forms (upper panel) and the RNA subunit of RNase MRP, *NME1* RNA (middle panel), were detected by RT-PCR. If RNase P has multimeric RNA subunits or exists in a complex with the closely related RNase MRP, the wild-type *RPR1* RNA or *NME1* RNA, respectively, were expected to copurify with S1-tagged *RPR1* RNA. Only S1-tagged *RPR1* RNA was found eluted from the beads. The faint band larger than S1-*RPR1* in the input is of unknown origin, and is likely an RT-PCR artifact. As a negative control, the presence of 5S rRNA in the input extract and eluate fractions was also shown by RT-PCR to be a negligible contaminant (lower panel).

although the previous chromatography conditions could easily have dissociated components if they were not tightly associated (Chamberlain et al., 1996; Lygerou et al., 1996). These results are also consistent with previous studies showing that antibodies to the unique protein subunits of RNase P and RNase MRP, Rpr2p and Snm1p, respectively, immunoprecipitate only their respective RNA subunit (Schmitt & Clayton, 1994; Chamberlain et al., 1998). As an additional negative control, RT-PCR was also used to demonstrate that the abundant 5S ribosomal RNA is not precipitated to a significant extent (Fig. 6, lower panel).

DISCUSSION

In this article, streptavidin-binding RNA aptamers were developed for which the binding could be displaced by biotin. This behavior is similar to that of a previously developed streptavidin-binding peptide, *Strep-tag* (Schmidt & Skerra, 1993), which was selected from a random peptide library. Proteins containing the *Strep-tag* at an accessible site can be purified by streptavidin-agarose and eluted with biotin or its analogs. An X-ray crystallographic study of *Strep-tag* shows the peptide binds at the same surface pocket for biotin (Schmidt et al., 1996). Although the binding site of our aptamers on streptavidin is not known, the ability to elute with biotin suggests that they might also bind at or near the biotin-binding sites.

The S1 aptamer was used successfully as an RNA affinity tag to isolate a ribonucleoprotein enzyme, RNase P. The aptamer tag has several advantages for this type of application. First, it has a high affinity for streptavidin. This property is very important to efficient purification of the tagged RNAs or RNPs, particularly when they are present at very low abundance. Affinity tags with low affinity to the corresponding ligand might fail to efficiently purify the tagged molecules (Wojczyk et al., 1996). Second, streptavidin-agarose beads are widely used and commercially available. This eliminates the necessity of preparing a special affinity resin, a potentially time-consuming process in which the quality of the matrix might be subject to batch-to-batch variation. Third, elution with biotin is done under mild conditions. Therefore, the activity of the aptamer-tagged RNP is preserved and the eluate is less contaminated with molecules that nonspecifically bind to the column support and might elute with high salt, pH changes, or the presence of denaturing agents. In addition, the biotin elution was found to be able to completely elute all of the bound aptamers from streptavidin (data not shown). This makes biotin elution different from other competitive elutions, such as glutathione for the GST tag, or imidazole for polyhistidine tag. Because the K_d of biotin to streptavidin is extraordinarily low, the binding is essentially irreversible, thus preventing the rebinding of the aptamers after they dissociate from streptavidin. It

should be noted that 2-iminobiotin, which has higher K_d , $\sim 10^{-7}$ M at pH 7.5 (Reznik et al., 1998), does not efficiently elute the S1 aptamer (data not shown). Finally, because of the small size of biotin (MW 244.31 Da), it can easily be removed from the eluate fractions by dialysis or ultrafiltration to obtain purified target RNAs or RNPs.

To successfully tag an RNA, the aptamer should be placed in a solvent-accessible region of the target RNA that does not interfere with structure and function of the RNP and is not removed during maturation of the RNP. Such information, if available, is very useful in choosing the site for tag insertion. Insertion into an accessible region can still fail due to misfolding, however. Our initial insertion of the S1 aptamer into an appropriate stem did not give an isolatable RNP until we adjusted the sequence surrounding the insertion to eliminate predicted interactions between the aptamer and the flanking *RPR1* sequences. It might be preferable in future applications to use only the minimal binding motif of the aptamer as a tag because it is almost 50% shorter, appears more tightly structured, and it has affinity to streptavidin comparable to the full-size form. Even the shorter form is not immune from folding problems, however. In recent experiments we inserted the minimal S1 aptamer in two positions in the pre-RPR1 leader sequence, and only one of the insertions gave a functional pre-RPR1 RNA even though computer modeling predicted both to fold correctly.

The procedure used here for isolating the aptamer-tagged RNase P directly from crude extracts with streptavidin-agarose beads was straightforward. Although these methods do not provide the 100,000-fold purification that is needed to completely purify RNase P (Chamberlain et al., 1998), complete isolation of more abundant RNPs might be possible in a single step, especially if more stringent washing conditions are used. It is possible to modify some purification steps to suit particular needs. It was found that the aptamer could bind well to streptavidin in binding buffer containing NaCl ranging from 100 to 400 mM (data not shown). Therefore, higher salt concentration might be used to reduce nonspecific binding, although this might dissociate some specific components that are loosely bound to RNPs. The length of the washing step can also be varied. We found that washing for 15–20 min was sufficient to separate the aptamer-tagged RNase P from the untagged form of RNase P or RNase MRP. However, bound complexes on streptavidin-agarose beads could be washed up to 2–3 h to reduce the amount of the background proteins with only a slight loss of RNase P. The advisability of shortening or lengthening the washing time might depend on several factors, including the stability of the target molecules, the desired cleanliness of the eluted product, and convenience.

In summary, the S1 streptavidin aptamer should provide a versatile tool for studying RNAs and RNPs. Because of the widespread uses of streptavidin in research, many commercially available preparations of streptavidin have been developed that could facilitate the detection, immobilization, and isolation of the target RNAs or RNPs.

MATERIALS AND METHODS

Yeast strains and materials

DNA oligonucleotides were synthesized by the University of Michigan DNA core facility. Streptavidin and streptavidin-agarose beads are from Gibco BRL. Egg-white avidin and D-biotin are from Sigma. *Taq* DNA polymerase, Sequenase, and T7 RNA polymerase were prepared as described by Tabor and Richardson (1989), Engelke et al. (1990), and Laing (1993).

S. cerevisiae strain JLY-1 (Lee et al., 1991) was originally generated from W3031A strain (*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) with *HIS3* disruption of *RPR1* gene and contains plasmid YCp50-*RPR1* expressing wild-type copy of *RPR1* RNA. In this study, the *HIS3* disruption of *RPR1* was replaced with *kan^R* gene.

Generating a random RNA library

To generate a random RNA library, single-stranded oligodeoxynucleotides (0.2 μ mol) were synthesized. They contain a randomized 40-nt central sequence flanked by defined primer-binding sites with the sequence as follows; 5'-AGTAATACGACTCACTATAGGGAGTCGACCGACCAGAA (N40) TATGTGCGTCTACATCTAGACTCAT-3'. A primer extension reaction of the oligodeoxynucleotides with 0.2 μ mol of the complementary 3' N40 primer (see Table 1) was done to generate a double-stranded DNA pool. The reaction was done at 60 °C for 4 h, using 200 U of *Taq* DNA polymerase in PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 0.2 mM of each dNTP). Approximately 60% of the single-stranded DNA could be converted into double-stranded DNA.

The DNA, which contains a T7 RNA polymerase promoter at the 5' end of the sequence, was then used as a template to generate an RNA library with T7 RNA polymerase. The transcription reaction was done at 37 °C for 1 h, in 40 mM Tris, pH 8.0, 20 mM MgCl₂, 25 mM NaCl, 1 mM spermidine, 20 mM DTT, and 4 mM of each NTP. The RNA was extracted twice with phenol/chloroform, precipitated with ethanol, and resuspended in 20 mL of 50 mM Tris, pH 8.0, 200 mM NaCl. It was then purified using Sephadex G-100, precipitated with ethanol, and resuspended in water. The resulting RNA sequences were 84 nt long (5'-GGGAGUCGACCGACCAGAA [N40] UAUGUGCGUCUACAUCUAGACUCAU-3') with a calculated complexity of the RNA library approximately 7×10^{16} different sequences.

In vitro selection of streptavidin-binding aptamers

RNA aptamers specific to streptavidin were obtained from the random RNA library, and the selection was done for a total of nine rounds. In the first six rounds of selection, streptavidin-agarose beads were used as a target. RNA (1–35 nmol or 1–25 μ M) was incubated with 30–45 μ L of streptavidin-agarose beads containing 5–8 nmol of streptavidin in binding buffer containing 50 mM HEPES, pH 7.4, 10 mM MgCl₂, and 100 mM NaCl. The binding step was done at room temperature for 1 h. The beads were then washed with 250 μ L of binding buffer, three to six times, for 1 min each. The bound RNA was eluted from the beads with elution buffer (8 M urea, 5 mM EDTA), and precipitated with ethanol. The RNA was reverse transcribed from a primer complementary to the known 3' sequence (3' N40 primer) with Superscript II (Life Technologies) as described by the manufacturer, and the cDNA was amplified with *Taq* DNA polymerase using T7 + 5' and 3' N40 primers (Table 1), for 5–10 cycles (95 °C 45 s, 60 °C 1 min, 72 °C 2 min). The PCR reaction was then used to generate RNA by in vitro transcription with T7 RNA polymerase. The resulting RNA was isolated from 10% polyacrylamide gel in 8 M urea, and used for subsequent rounds of selection. Starting from Round 3, a subtraction step with Sepharose 4B was introduced to remove RNAs that have affinity toward the bead matrix instead of streptavidin. This step

TABLE 1. Primers used in the study.

Primers for in vitro selection	
T7 + 5'	5'-AGTAATACGACTCACTATAGGGAGTCGACCGACCAGAA-3'
3' N40	5'-ATGAGTCTAGATGTAGACGCACATA-3'
Primers for constructing S1-RPR1	
5'RPR1-S1	5'-GGGTAAATGTCATATGAAAAGGGAGTCGACCGACCAG-3'
3'RPR1-S1	5'-GGGCCAATGCCATATGTTTATGAGTCTAGATGTAGA-3'
RPR1-SacII	5'-GGGGCTAACCGCGGGGTAGTTACATATACTAG-3'
RPR1-ApaI	5'-GGAACCTAAGGGCCCCCAACAAATTCAATAGTTACTATCGC-3'
Primers for RT-PCR amplification	
RNP100	5'-ACTAGTCCCGGGAATCCATGGACCAAGAATAGTAAG-3'
RNP53	5'-GACGTCTTACGATTCGAC-3'
Y5'MRP	5'-ACTAGTCCCGGGAATCCATGACCAAGAATCGTCACAAATCGAAGCTTACAAATGGAGTAAAATTTCTTTAC-3'
Y3'MRP	5'-ACTAGTCCCGGGAATCCATGGACCAAGAATAGTAAG-3'
Yeast5'5S	5'-TAATACGACTCACTATAGGTTGCGCCATATCTACCAGA-3'
Yeast3'5S	5'-TGCAGCACCTGAGTTTCGCGTATGGTC-3'

was done by preincubating RNA with 30–200 μ L of Sepharose 4B at room temperature for 1 h before the beads were spun down, and the supernatant was used in the binding step with streptavidin-agarose beads as described.

In the last three rounds of selection (Rounds 7–9), streptavidin aptamers were enriched using electrophoretic mobility shift. Radiolabeled RNA (~300,000 cpm or 5–10 pmol), generated with T7 RNA polymerase in the presence of α - 32 P-UTP, was incubated with 20 μ g (0.3 nmol) of streptavidin in binding buffer at room temperature for 1 h. The sample was then loaded into a nondenaturing 6% polyacrylamide, and run at 110 V for 2 h; the binding buffer was used as both the gel and running buffers. The gel was exposed to an X-ray film with an intensifying screen at -80°C for 1–12 h. The shifted RNA band was excised and soaked in elution buffer (0.1 M sodium acetate, 1 mM EDTA, and 0.2% SDS) at 37°C overnight. The RNA was then used for RT-PCR and in vitro transcription to generate RNAs for the next round of selection.

The PCR products from the final round were cloned into pGEM-T vector (Promega). Each individual aptamer was generated from plasmid clones by PCR amplification with the T7 + 5' and 3' N40 primers, followed by in vitro transcription with T7 RNA polymerase. The affinity of each aptamer to streptavidin was tested by electrophoretic mobility shift assays. The positive clones were sequenced by dideoxy chain termination methods, and their sequences were analyzed.

Electrophoretic mobility shift assays

A radiolabeled aptamer, 5,000 cpm or 0.1 pmol, was incubated with 1 pmol of streptavidin in binding buffer at room temperature for 1 h (5 μ L total volume). Two microliters of 30% glycerol in binding buffer were then added to the reactions and the samples were loaded into 6% nondenaturing polyacrylamide gel in binding buffer and run at 110 V for 2 h. The gels were dried and radioactive bands were visualized using a PhosphorImager (Molecular Dynamics). Binding of radiolabeled RNA (5 nM) to different amounts of streptavidin (0–4 μ M) was performed to determine the dissociation constants of the aptamers as described by Soukup et al. (1996).

To study the effect of biotin on the aptamer binding, 20 nM radiolabeled aptamer was incubated with 250 nM streptavidin that had previously been incubated with binding buffer $\pm$ 2 mM biotin for 15 min at room temperature for 1 h. To study the effect of biotin on aptamer dissociation from RNA/streptavidin complexes, the complexes were preformed by incubating 20 nM radiolabeled aptamer with 250 nM streptavidin in binding buffer at room temperature for 1 h. The complexes were then incubated with binding buffer containing 2 mM biotin for different periods (0–120 min) or with various concentrations of biotin (0–2 mM) for 2 h. The reactions were analyzed on nondenaturing polyacrylamide gels as described above.

Binding to streptavidin versus egg-white avidin

To test whether egg-white avidin would bind to the S1 aptamer, 15 nM 32 P-labeled S1 aptamer was preincubated in binding buffer at room temperature for 15 min with increasing concentrations of either streptavidin or egg-white avidin. This

was followed by incubation for 60 min with streptavidin-agarose beads and measurement of bound aptamer. Binding was 50% inhibited by 30 nM streptavidin, but was not inhibited 50% by egg-white avidin concentrations greater than 2.0 μ M (data not shown).

Constructing *RPR1* RNA containing S1 aptamer

The S1 aptamer was inserted into *RPR1* gene using in vitro overlap-extension PCR (Ling & Robinson, 1997). Briefly, a PCR fragment was generated from a plasmid containing the S1 aptamer by using 5'RPR1-S1 and 3'RPR1-S1 primers (Table 1). The resulting PCR product contained the aptamer sequence flanked by *RPR1* sequences at both ends. The PCR strands were then used as large primers in overlap-extension PCR with either RPR1-SacII or RPR1-ApaI primers, with an *RPR1* gene as a template. The final PCR product contained *RPR1* sequence from positions -382 to $+465$ with the full-length S1 aptamer inserted between positions $+133$ and $+140$ in the original *RPR1* sequence. It was then purified, cleaved with SacII and ApaI, and cloned into the SacII-ApaI sites of pRS315. NdeI restriction sites were also generated, flanking the inserted aptamer to facilitate future removal of the aptamer or incorporation of a new RNA tag (Fig. 4A). In addition, adenines were added on each side of the aptamer to help the aptamer fold correctly. The initial construct without the adenine stretches was found to bind less efficiently to streptavidin-agarose beads compared with the one with the adenine stretches. From the predicted secondary structure of the aptamer-tagged *RPR1* RNA (Mathews et al., 1999), it was found that the adjacent sequences of *RPR1* RNA were likely to cause misfolding of the aptamer. The additional adenine spacers were predicted to cure this, helping the aptamers to fold correctly and independently from the adjacent sequences (Fig. 4A).

Either pRS315 containing wild-type *RPR1* (Pagán-Ramos et al., 1996) or the aptamer-tagged *RPR1* was transformed into JLY-1 yeast strains, and YCp50-*RPR1* was removed using 5-fluoroorotic acid. The resulting strains, wild-type or S1-*RPR1*, were subsequently analyzed for their growth phenotypes and *RPR1* RNA processing to confirm that the aptamer-tagged *RPR1* could complement the loss of the wild-type *RPR1*.

Isolation of RNase P using streptavidin-agarose beads

To prepare crude extracts from the wild-type and S1-RPR1 strains, 25–1,000-mL cultures of yeast strains in YPD were grown to 2×10^7 cells/mL. The cells were harvested and washed twice with water. The pellets were resuspended in 0.4–5 mL of a lysis buffer (50 mM HEPES, pH 7.4, 10 mM MgCl_2 , 100 mM NaCl, 1 mM DTT, 0.1% Triton-X100, 10% glycerol) containing protease inhibitors Complete® (Roche). The cells were vortexed with 0.5–10 g of acid-washed glass beads, size 425–600 μ m (Sigma), at room temperature for a total of 30 min; the samples were placed on dry ice briefly every 2 min to keep their temperature low, around 4°C . Debris was removed by centrifugation twice at $14,000 \times g$ for 5 min at 4°C . The protein concentration was determined using

the Micro Bicinchoninic acid Assay (Pierce). All subsequent steps were performed at 4 °C.

To isolate RNase P, soluble cell extract containing 2.5 mg protein was preincubated with 12.5 μ g of egg-white avidin (5 μ g of avidin/mg protein) for 10 min. The mixtures were then incubated with 25–50 μ L of streptavidin-agarose beads for 1 h; the binding reactions were performed in the lysis buffer condition in a total volume of 125 μ L. The beads were spun down to remove the supernatant and washed with 1.5 mL of the lysis buffer eight times, for 10 min each. The beads were transferred to Ultrafree-MC centrifugal filter units –0.45 μ m size (Millipore) and further washed with 250 μ L of lysis buffer three times for 5 min each. Elution was accomplished by incubating with 100 μ L of the lysis buffer containing 5 mM biotin for 0.5–1 h. The eluates were quickly frozen in dry ice and kept at –80 °C for later analyses.

The samples from the input and eluate fractions were analyzed for RNase P activity and the presence of *RPR1* RNA. RNase P activity assay was done using ³²P-labeled pre-tRNA^{Asp} substrate as described by Hollingsworth and Martin (1987). The reactions were done in the lysis buffer and incubated at 37 °C for 15 min. The products were analyzed on 10% denaturing polyacrylamide gel in 8 M urea. The presence of *RPR1* RNA were analyzed by northern blot analysis with a radiolabeled complementary RNA probe as described by Tranguch and Engelke (1993). The blots were visualized using a PhosphorImager and the band intensity was quantitated using IPLab Gel analysis software (Signal Analytics). The purification yield of RNase P was calculated as a percentage of the amount of *RPR1* RNA recovered in the eluate over that of the *RPR1* RNA in the input fraction.

Testing complex formation with RNase MRP and multiple *RPR1* copies

JLY-1 strain was transformed with pRS315 containing the aptamer-tagged *RPR1*, and plated on SC –Leu –Ura media. The resulting yeast strain had both wild-type *RPR1* RNA from YCp50-*RPR1* and the aptamer-tagged *RPR1* RNA from the pRS315 plasmid. The crude extract was prepared from yeast cultures grown in SC –Leu –Ura. The extract containing 1.5 mg protein was preincubated with avidin, and later incubated with streptavidin-agarose beads as described above. The beads were washed with 250 μ L of lysis buffer for 3 min, five times, and eluted with 100 μ L of lysis buffer containing 5 mM biotin. The input and eluate fractions were analyzed for the presence of *RPR1* RNA, *NME1* RNA, and 5S rRNA by RT-PCR. RNP100 and RNP53 primers were used to generate a PCR product spanning from positions 80 to 327 of *RPR1* RNA, which includes the aptamer insertion region. The products are 248 bp for wild-type *RPR1* RNA and 345 bp for the aptamer-tagged *RPR1* RNA. Y5'MRP/Y3'MRP and Yeast5'S/Yeast3'S primer pairs were used to generate PCR fragments encompassing positions 1 to 336 of *NME1* RNA, and positions 1 to 117 of 5S rRNA, respectively.

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