tRNA prefers to kiss

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Six RNA aptamers that bind to yeast phenylalanine tRNA were identified by in vitro selection from a random-sequence pool. The two most abundantly represented aptamers interact with the tRNA anticodon loop, each through a sequence block with perfect Watson–Crick complementarity to the loop. It was possible to truncate one of these aptamers to a simple hairpin loop that forms a classical ‘kissing complex’ with the anticodon loop. Three other aptamers have nearly complete complementarity to the anticodon loop. The sixth aptamer has two sequence blocks, one complementary to the tRNA T loop and the other to the D loop; this aptamer binds better to a mutant tRNA that disrupts the normal D-loop/T-loop tertiary interaction than to the wild-type tRNA. Selection of complements to tRNA loops occurred despite an attempt to direct binding to tertiary structural features of tRNA. This serves as a reminder of how special the RNA–RNA interactions are that are not based on complementarity. Nonetheless, these aptamers must present the tRNA complement in some special structural context; the simple single-strand complement of the anticodon loop did not bind tRNA effectively.

Keywords: in vitro selection/kissing complex/RNA–RNA interactions/RNA stem–loop/tRNA

Introduction

Intermolecular interactions between the major classes of RNAs play fundamental roles in gene expression. Examples are mRNA–tRNA, rRNA–mRNA (Shine–Dalgarno interaction), tRNA–rRNA, RNase P RNA–pre-tRNA, snoRNA–pre-rRNA, snRNA–pre-mRNA and snRNA–snRNA. Other RNAs also make biologically relevant RNA interactions of a more specific nature, e.g. the HIV-1 genomic RNA contacts a second identical partner molecule during packaging into the viral particle, and upon infection interacts with cellular tRNA533 to prime reverse transcription (Barat et al., 1989; Dardel et al., 1998). Most of these interactions are based on sequence complementarity of at least three contiguous base pairs, a clear exception being recognition of the pre-tRNA substrate by the RNase P ribozyme, which depends mainly on tertiary structure of the substrate (Carrara et al., 1995; Yuan and Altman, 1995). RNA–RNA interactions not based on complementarity are also found in intramolecular tertiary interactions. While the classical tertiary interactions within tRNA show a distributed point-to-point pattern (Kim, 1979), other RNAs can have rather extensive region-to-region interactions that do not employ base-pairing. An example is binding between the P1 stem and the single-strand J8/7 region of the Tetrahymena group I intron, in which many 2′-OH groups of P1 become hydrogen-bonded; this interaction is sufficient to allow a separate P1 stem to function in trans with the rest of the ribozyme (Doudna et al., 1989; Szewczak et al., 1998).

Many proposals have been made for an RNA world that attained great complexity prior to the advent of coded protein synthesis (Orgel, 1986; Benner et al., 1989; Gesteland et al., 1999). One presumes that both sequence-specific and structure-specific RNA–RNA interactions would play useful roles in such a world. Indeed, by most accounts, the modern tRNA–RNase P RNA interaction, based on structure more than complementarity, has descended from that time.

How readily could the various types of RNA–RNA interaction evolve? The in vitro selection approach can be employed to address experimentally how prevalent different kinds of binding partners are for various target RNAs in unbiased searches of RNA sequence space. We have begun such an investigation by selecting RNA aptamers from a random sequence pool that binds efficiently to tRNAPhc. We find that complementarity to loops (usually the anticodon loop) in the tRNA target is likely to explain much of the aptamers’ interaction, but also that the aptamers must present these complements in some special way; the simple single-strand complement at the anticodon loop does not bind tRNA effectively. One aptamer is shown to form a classical ‘kissing complex’ with full base-pairing of complementary hairpin loops.

Results

Aptamers to tRNA have complementarity to tRNA loops

We employed in vitro selection to identify RNA sequences that bind with high affinity to a T7 transcript corresponding to phenylalanine tRNA of Saccharomyces cerevisiae (Figure 1). RNA aptamers were selected from a pool with an 80 nucleotide (nt) region of fully random sequence, flanked by constant-sequence regions for reverse transcription and PCR, which contained ∼3.4 × 1014 independent sequences. Selection was by tRNAPhc affinity chromato-
with free tRNAPhe transcript. The enriched pool was affinity chromatography on the tRNA resin, affinity eluting a total of 11 unique sequences were found (Figure 2B). Two unique sequences (B2 and B3) were sequenced. Individual molecules from the pool were cloned, and 40 progressing to 55% binding in the tenth cycle (Figure 2A).

Binding sites of tRNA on the B2 and B3 aptamers are disrupted in one of the mutants are indicated, as is the position of the fluorescent base of the native tRNA.

Fig. 1. Secondary structure of the T7 transcript of yeast tRNAPhe and two mutant versions used in this study. Two tertiary interactions that are disrupted in one of the mutants are indicated, as is the position of the fluorescent base of the native tRNA.

tRNA, disrupted in tertiary structure, which had been employed with the intention of counter selection (Table I). The two most abundant aptamers bound with equalefficiency to mutant and wild-type tRNA resins. Thus, our attempt at counter selection did not result in dependence on tRNA tertiary structure. Indeed the binding of aptamer B1 to the mutant tRNA resin was substantially more efficient than to the wild-type tRNA resin. Although the aptamers bind well to the mutant tRNA resin, substantial fractions do flow through, and this high flow-through must have been responsible for the apparently limited effectiveness of counter selection during in vitro selection.

Inspection of primary sequences from the pool suggested complementarity to tRNA loops as a basis for aptamer binding (Figure 2B) and allowed sorting into three classes. The singly represented sequences, which exhibited ineffective binding, had no blocks longer than 4 nt complementary to any of the three loops in tRNAPhe. One aptamer, B1, has two regions of complementarity to tRNAPhe, one to the T loop and the other to the D loop. This was the aptamer that bound the mutant tRNA better than the wild type. The other five aptamers contain a block of perfect Watson–Crick complementarity to the first six or all seven nucleotides of the tRNAPhe anticodon loop (Figure 2B). The two most abundantly represented aptamers, B2 and B3, were of this class and were characterized further.

Aptamers affect the anticodon loop in tRNA
To address the hypothesis that B2 and B3 interact with the tRNA anticodon loop, a classical analytical feature of the natural yeast phenylalanine tRNA was employed—the hypermodified fluorescent base wybutine of the anticodon loop (Figure 1). The fluorescence of this base has been used extensively to monitor alterations of anticodon loop structure and interactions with other molecules (Robertson et al., 1977; Fairclough et al., 1979; Claesens and Rigler, 1986). We found that addition of either the B2 or B3 aptamer to native tRNAPhe caused a substantial increase in its fluorescence emission (Figure 3B and C). In contrast, no fluorescence change was detected when RNA B13, an ineffective ligand (Table I), was added to tRNAPhe (Figure 3D). The aptamer-induced increase of fluorescence emission suggests a change in anticodon loop conformation, as would be expected from base pairing and increased stacking upon interaction with the complementary aptamer segment. This experiment suggests further that the numerous nucleotide modifications of native tRNAPhe do not prevent aptamer binding.

The effect of the aptamers on tRNAPhe fluorescence could in principle be an indirect effect transmitted to the anticodon upon binding at a different tRNA site. Instead, direct interaction of the aptamers with the anticodon loop was indicated by the failure of affinity chromatography on a resin bearing a mutant tRNAPhe, with four central nucleotides of the anticodon loop altered (Figure 1; Table I). This resin was active, being bound very efficiently by a truncated form of B3 that had been altered to restore complementarity to the anticodon mutant tRNA (see below).

tRNA affects the complementary segments in aptamers
The binding sites of tRNA on the B2 and B3 aptamers were determined by footprinting, using nucleases specific
Selection of tRNAPhe aptamers

Fig. 2. Selection of tRNA aptamers. (A) tRNA binding activity of the RNA pool through the course of in vitro selection. (B) Sequences recovered from the final pool. Sequences of the originally randomized block are shown; additional sequences common to all aptamers are GGGAUAUCGCGUGUCG at the 5'-end and GUCCGUUCGGGAUCCUC at the 3'-end. Number of occurrences among the 40 clones sequenced are reported in parentheses. Capitalized blocks are complementary to a loop in tRNA Phe, with at least six contiguous Watson–Crick base pairs; underlining marks proposed base-pairings that abut the 3'-end of the tRNA complement (note a proposed G–A pair for B3; see Figure 7).

Table I. Binding efficiency of aptamers to tRNA resins (recovery as percentage of load)

<table>
<thead>
<tr>
<th>tRNAWT</th>
<th>Tertiary mutant</th>
<th>Anticodon mutant</th>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Full-length RNA</td>
<td></td>
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<tr>
<td>B2</td>
<td>66</td>
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<tr>
<td>B3</td>
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<td>B4</td>
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<td>B7</td>
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<tr>
<td>B10</td>
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<tr>
<td>B11</td>
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</tr>
<tr>
<td>B13</td>
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</tr>
<tr>
<td>B14</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Truncated RNA</td>
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<td></td>
</tr>
<tr>
<td>S3</td>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td>S3v</td>
<td>0</td>
<td>87</td>
</tr>
<tr>
<td>L3</td>
<td>6</td>
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Table II. Dissociation constants of tRNA-binding RNAs

<table>
<thead>
<tr>
<th>tRNAWT</th>
<th>Tertiary mutant</th>
<th>Anticodon mutant</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Full-length RNA</td>
<td></td>
<td></td>
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<tr>
<td>B2</td>
<td>12 ± 1.2</td>
<td></td>
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<tr>
<td>B3</td>
<td>26 ± 1.4</td>
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<tr>
<td>Truncated RNA</td>
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<td></td>
</tr>
<tr>
<td>S3</td>
<td>21 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>S3v</td>
<td>1 ± 0.25</td>
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</table>

*Dissociation constants were measured three times by isocratic competitive affinity chromatography with the indicated standard deviations.

to single-strand (T1 and S1 nuclease) or double-strand (V1 nuclease) regions (Figure 4). In the absence of tRNA, the segments complementary to tRNA are the longest single-strand regions in either aptamer, being susceptible to T1 and S1 and resistant to V1 (not shown for B2). In the presence of tRNAPhe, this pattern is reversed; T1 and S1 cleavages are suppressed and V1 cleavages are induced in the complementary segments. Thus, tRNA converts the complementary block in these aptamers from the single-strand to the double-strand state. Such reversal of nuclease sensitivity upon binding is a hallmark of the classical
kissing complex, formed by pairing of complementary hairpin loops in the Col E1 plasmid RNAs I and II (Eguchi and Tomizawa, 1991).

The sequence of B3 is compatible with formation of a hairpin stem–loop, with the sequence complementary to tRNA presented in the loop, and thus a kissing complex can be proposed for the B3–tRNA interaction. We present conclusive proof of this hypothesis below, but here we note that the nuclease sensitivity pattern of free B3 is consistent with the hairpin stem–loop structure. Furthermore, details match those of the RNA I–RNA II kissing complex (Eguchi and Tomizawa, 1991). Specifically, susceptibility of B3 to V1 nuclease is altered by tRNA in the stem proximal to the complementary loop, reducing cleavage on the 5'H11032 side and enhancing cleavage on the 3'H11032 side (Figure 4B, lane 6).

Aptamer truncation

In order to identify smaller versions of B2 and B3, the 5' and 3' boundaries of the active forms were mapped. Aptamers were labeled at either the 5'- or 3'-end and subjected to partial alkaline hydrolysis (Pan and Uhlenbeck, 1992). The resulting RNA fragments were loaded onto a tRNA column, with inactivated forms flowing through and fragments retaining tRNA-binding activity specifically eluted. These fractions were then analyzed by polyacrylamide gel electrophoresis.

Very little truncation of B2 was possible. Only 11 nt could be removed from either end without inactivating the aptamer (data not shown). In contrast, most of the B3 molecule was dispensable. The 5'- and 3'-boundaries that were identified (Figure 5) map aptamer activity to a mere 19 nt segment containing the complementary sequence shown above to bind tRNA. This segment, S3, was prepared (with a short 5' leader) by transcription of synthetic oligonucleotides (Figure 6). It was highly active in tRNA affinity chromatography, and the dissociation constant measured by isocratic elution was the same as for the full-length B3 (Table II). This small aptamer has a clear propensity to form a stem–loop structure, with the segment complementary to tRNA contained in the loop. The even shorter RNA molecule L3, consisting of merely the segment complementary to tRNA Phe without a flanking stem (Figure 6), did not bind efficiently to the tRNA column (Table I).

The small size of S3 facilitated a proof, by compensatory base-pair change, that the aptamer base pairs with the tRNA Phe anticodon loop. The variant S3v of the minimal aptamer was prepared (Figure 6), with four base changes in its loop that compensate for the anticodon loop changes in the mutant tRNA shown in Figure 1. S3v did not bind to the wild-type tRNA shown (Table I). Affinity was 20-fold higher with the mutant partners (S3v and anticodon mutant tRNA) than for the original interaction exemplified by S3 and wild-type tRNA. Energy minimization algorithms predict intraloop base-pairing in S3 that is not shared by S3v (Figure 6; Zuker, 1989), which may be responsible for the differing affinities for the respective partners. However, nuclease digestion of the intact B3 gave no indication that these intraloop base pairs form to any great extent (Figure 4B).
Selection of tRNA\textsuperscript{Phe} aptamers

Fig. 4. Enzymatic probing of the B2 (A) and B3 (B) RNAs. 5'-\textsuperscript{32}P-labeled RNAs were partially digested with RNases S1, T1 or V1 in the presence (+) or absence (-) of 5 \mu M tRNA\textsuperscript{Phe}. Aliquots of enzymatic digestions were loaded on a denaturing 12% polyacrylamide gel, along with the corresponding partial alkaline hydrolysate (OH–) and denaturing partial RNAse T1 digest (G). Square parentheses mark the segments complementary to the tRNA\textsuperscript{Phe} anticodon loop.

Discussion

The B3 aptamer was shown to consist essentially of a simple stem–loop that forms a 'kissing' complex; its loop base-pairs with perfect Watson–Crick complementarity to the entire 7 nt anticodon loop of tRNA. A natural kissing complex formed between Col E1 plasmid RNAs I and II provides an excellent model for the B3 aptamer–tRNA complex (Eguchi and Tomizawa, 1991), with several bases at corresponding positions in the two complexes being identical or preserving pyrimidine/purine character, especially at key positions near the loop–stem junctions (Figure 7). RNA I regulates the plasmid replication priming activity of RNA II, and the Col E1 Rom protein modulates this regulation by binding the RNA I–RNA II complex. Rom recognizes unique structural features of this kissing complex, but without sequence specificity. It binds with equal affinity to several variant complexes with fully base-paired loop–loop helices of 6, 7 or 8 bp (Eguchi and Tomizawa, 1991). Two structures of kissing complexes capable of binding Rom have been solved by nuclear magnetic resonance (NMR) (Chang and Tinoco, 1997; Comolli et al., 1998; Lee and Crothers, 1998). Both show complete base-pairing and continuous stacking of the three helices. Spanning of the loop–loop–helix by single phosphodiester bonds at each 5' end is accomplished by strong bending in the center of the helix that brings its ends closer to each other, and by unusual helical parameters involving base pairs on either side of the helix junctions. Structural information is also available for other kissing complexes (of HIV-1 genomic RNA dimerization and between anticodons of tRNA) (Grosjean et al., 1976; Moras et al., 1986; Dardel et al., 1998; Mujeeb et al., 1998), but these are somewhat less relevant to the B3–
intrahelical case where G–A imino pairs form), the G
and Puglisi, 1998). In all these cases (except for one
et al (SantaLucia and Turner, 1993; Biou et al.
the phosphodiester 3
pairings within helices, and in GNRA tetraloops where
hammerhead ribozyme, in GA–GA or GAAA–GAAA
D stem–loop, in the HIV-1 A-rich RNA loop, in the
following the 3
helices capped by an opposed G–A pair (with the G
information is available for several RNA Watson–Crick
we propose forms a sheared G–A base-pair. Structural
pairs. The most important difference between the RNA I–
randomized, and explores other potential closing base
antisense transcription of the same DNA segment. For
fully loop-paired kissing complex, is also a model for
antisense RNA interaction; the two RNAs arise from
Fig. 7. Complementarity-based complexes. Putative aptamer-tRNAPhe
complex structures are compared with a portion of the Col E1 plasmid
RNA I–RNA II complex (Eguchi and Tomizawa, 1991), drawn in the
continuously stacked form observed in an NMR study of a derivative
of the RNA I–RNA II complex (Lee and Crothers, 1998). Bases that
preserve purine or pyrimidine character relative to the Col E1 complex
are capitalized and identical bases are in bold. Non-Watson–Crick base
pairs are marked by dots.

tRNA structure because they do not involve all loop bases
in pairing.

A variant of the RNA I–RNA II complex, with inverted loop
sequences, has 350-fold increased affinity (Eguchi
Tomizawa, 1991). The effect was attributed mainly
to the identity of the terminal base-pairs of the loop–loop
stem (Gregorian et al., 1995). We also identified a loop-
alternated variant of the S3 aptamer–tRNA complex with
20-fold increased affinity, but in this case only central
loop base pairs were altered. We suspect a different
basis for this affinity increase: faster association due to
elimination of a propensity for intraloop base-pairing in
the original aptamer (Figure 6).

The RNA I–RNA II complex, the prototype for the
fully loop-paired kissing complex, is also a model for
antisense RNA interaction; the two RNAs arise from
antisense transcription of the same DNA segment. For
this reason, only Watson–Crick pairs could be expected
for closing the natural hairpin loops, and non-Watson–
Crick closing base-pairs have not been tested in kissing
complexes. In vitro selection starts with all positions fully
randomized, and explores other potential closing base
pairs. The most important difference between the RNA I–
RNA II and aptamer–tRNA complexes may be the closure
of the aptamer hairpin stem with a G–A opposition, which
we propose forms a sheared G–A base-pair. Structural
information is available for several RNA Watson–Crick
helices capped by an opposed G–A pair (with the G
following the 3’ end in the helix), that occur at a tRNA
D stem–loop, in the HIV-1 A-rich RNA loop, in the
hammerhead ribozyme, in GA–GA or GAAA–GAAA
pairings within helices, and in GNRA tetraloops where
the phosphodiester 3’ to the G spans two stacking levels
(SantaLucia and Turner, 1993; Biou et al., 1994; Pley
et al., 1994; Scott et al., 1995; Baeyens et al., 1996;
Jucker et al., 1996; Wu and Turner, 1996; Viani Puglisi
and Puglisi, 1998). In all these cases (except for one
intrahelical case where G–A imino pairs form), the G
and A form a sheared pair, and the amino group of the
adenine makes a hydrogen bond to the 2’-OH of the
opposed G. A hydrogen bond between the G amino and
a nonbridging oxygen of the phosphate 5’ to the A is also
frequently observed. The effect is to narrow the helix at
this pair and send the protruding 3’ phosphodiester from
the G more parallel to the helix than occurs from Watson–
Crick pairs. These features at a hairpin-closing sheared
G–A pair may improve kissing complex formation relative
to a closing Watson–Crick pair.

The B2 aptamer also binds the anticodon loop using a
sequence with perfect Watson–Crick complementarity. As
for B3, this complement is unpaired in the free aptamer
and base-paired in the complex. However the sequence
flanking the complement in B2 does not suggest a strong
hairpin. Instead, energy minimization algorithms repeat-
edly produce a stem–loop immediately downstream of the
complement (underlined in Figure 2), which could perform
the 3’ stacking function of the hairpin stem in a kissing
complex (Figure 7). Even if this proposed complement-
flanking stem feature is part of B2, its striking resistance
to truncation shows that other regions of B2 are required
for aptamer activity, even if only for the negative function
of preventing alternative folds. Immediately downstream
stem–loops, but not surrounding hairpin loops, can be
proposed for the three aptamers (B4, B6, and B7) that
contain the perfect 6 nt complement to the 3’ portion of
the anticodon loop. These three and B2 all have a
G-pyrimidine sequence following the proposed stem
(Figure 2).

We found that the short linear RNA L3, containing the
7 nt anticodon complement present in B2 and B3, did not
bind the tRNA resin efficiently. This could also be
surmized from the boundary experiments; boundaries did
not reach the anticodon complement in either aptamer
from either direction. This stands in striking contrast to
work with the RNA I–RNA II complex, where the linear
complement bound the RNA II stem–loop with higher
affinity than did the RNA I stem–loop (Eguchi
Tomizawa, 1991); however, this linear molecule actually
had 10 nt complementary to the stem–loop and may have
denatured the stem–loop to form the 10 bp complex. We
also note that binding of native tRNAPhe by the RNA
uUCUUUCu with 5 nt complementarity (upper case) had a
Kd of only 10^-4 M at 12°C (Labuda et al., 1985). The
observed inefficient binding of L3 implies that our system
demands something more than simple possession of an
unpaired region complementary to the anticodon loop.
Instead the aptamers must present the complement in a
favorable context. We suggest that a stem immediately 3’
to the anticodon complement (either a surrounding hairpin
stem, or a downstream stem–loop) provides a stacking
face in these aptamers (Figure 7). The anticodon stem
might then provide a stacking face for the other end of the
anticodon loop–aptamer helix.

The B1 aptamer shows perfect complementarity to both
the D and T loops of tRNAPhe (except for one wobble
pair in each pairing). This matches its more efficient
binding of the tertiary mutant tRNAPhe than the wild type.
Although the mutant loses one D-loop Watson–Crick pair
with the aptamer, its disruption of tertiary structure frees
both D and T loops for pairing. On the wild-type tRNAPhe
resin, B1 must rely on the presumably small fraction of
incompletely folded tRNA (although the aptamer may actively unfold the tRNA transcript). Pairing by B1 at one of these two tRNA loops would then leave the other loop free so that the second pairing could occur rapidly.

We used a T7 transcript corresponding to the yeast tRNA^{Phe} as both the immobile phase and eluant in the affinity chromatography system of the selection. Several studies using this same transcript have indicated that it reproduces structural features and many interactions of the native tRNA (Hall et al., 1989; Harrington et al., 1993). Our data indicate that the several modified nucleotides of the native tRNA did not prevent binding of the aptamers. In such selection experiments, one is faced with choosing a site on the target molecule at which to immobilize it, and must sacrifice that potential aptamer binding site. For example, our choice of the 3’ end of the tRNA for immobilization may have precluded selection of a mimic of RNase P RNA.

In vitro selection is an excellent experimental system for studying RNA–RNA interaction. In the context of the group I intron ribozyme, in vitro selection has shown that functional RNA domains can be compensated for after deletion (Green and Szostak, 1992) or replaced with unrelated but rare sequences (Williams et al., 1994), and has been used to study triple helical regions and (Green and Szostak, 1994) GNRA tetraloop–receptor RNA interactions (Costa and Michel, 1997). One study particularly active unfolded the tRNA transcript). Pairing by B1 at one of these two tRNA loops would then leave the other loop free so that the second pairing could occur rapidly.

Materials and methods

RNA synthesis

An initial pool of $4 \times 10^{15}$ individual DNA molecules, 114 nt long, containing 80 nt of random sequence flanked by defined regions at each end, was synthesized chemically and purified on a denaturing polyacrylamide gel. The 5’ PCR primer, 5’-TAATACTGACTCTACTATA-GGGGATCCGGGTTGCGC, contained the sequence of the T7 RNA polymerase promoter, and the 3’ PCR primer was 5’-GAGGATCCGG-AACGGAC. It was found that 8.4% of the random oligonucleotides could be fully extended by T7 DNA polymerase, giving an effective complexity of the DNA pool of $3.4 \times 10^{14}$ different sequences. Transcript templates were created by PCR amplification of degenerate oligos, followed by a fresh single PCR cycle to rectify heteroduplex accumulated during the last PCR cycles (Green et al., 1991). The RNA pool was synthesized by T7 RNA polymerase transcription and purified on denaturing polyacrylamide gels.

Genes for the wild-type and mutants of yeast tRNA^{Phe}, under control of the T7 promoter, were assembled from a set of eight oligodeoxynucleotides (Reyes and Abelson, 1987), and cloned into the pUC19. To synthesize tRNA^{Phe}, plasmid DNAs were cleaved with BstNI restriction enzyme transfected with T7 RNA polymerase and purified on denaturing polyacrylamide gels. S3 and S3v RNA were synthesized by T7 RNA polymerase transcription of partially duplex synthetic DNA templates (Milligan et al., 1987).

Selection procedure

Selection was performed using a tRNA^{Phe} affinity matrix containing 3 mM tRNA. The tRNA^{Phe} was oxidized at the 3’ terminus as described by Fahnestock and Nomura (1972). Excess periodate was removed by repeated precipitation on the tRNA with 2.5 vol. of ethanol at -20°C. Washed, oxidized tRNA was dissolved in 0.1 M sodium acetate pH 5.0 at a concentration of 4 μM. Coupling of oxidized tRNA to adipic acid dihydrazide-Sepharose was accomplished by mixing and agitating gently at 4°C for 12 h. The resulting tRNA-Sepharose was washed repeatedly with 2 M KCl to remove all non-covalently bound material. After the high-salt wash, the tRNA–agarose was equilibrated with binding buffer. In the first two cycles, the RNA pool was passed through a counter-selective precolumn of quenched adipic acid dihydrazide agarose, to remove aptamers recognizing the matrix per se. Beginning with the third cycle, the counter-selective precolumn matrix was replaced with a resin bearing a mutant tRNA^{Phe} disrupted in tRNA tertiary structure. 32P-labeled RNA was denatured in water for 4 min at 70°C and cooled to room temperature for 10 min in column buffer before being loaded onto the affinity column pre-equilibrated with 50 vol. of column buffer (0.25 M NaCl, 50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 2 mM spermidine, 0.2 mM EDTA). In the first round of selection, 30 nmoL of the initial pool, corresponding to 48 copies of individual sequences, were loaded on a 1 ml tRNA–agarose column. In subsequent rounds of selection, 1–3 nmoL of RNA were applied to 0.3-ml columns. RNA was allowed to bind to the column for 20 min, and unbound molecules were then washed extensively with column buffer until the level of radioactivity had reached a plateau. After the wash step, the RNA molecules were eluted with 10 μM tRNA^{Phe}. The eluted RNAs were extracted with phenol–chloroform, ethanol precipitated in the presence of 20 μg of glycogen, and quantified by Cerenkov counting. Water-dissolved RNA was reverse transcribed and amplified by PCR. The PCR products from round 10 of the selection were cloned by restriction enzyme cleavage at the EcoRI and BamHI sites in the 5’ and 3’ primer regions, respectively, and ligated into the pUC 19 vector.

Minimal sequence requirements for tRNA binding

The RNAs were labeled at either the 3’- or 5’-end with 32P and then partially digested under mild alkaline conditions (Pan and Uhlenebeck, 1992). Active aptamer fragments were purified as during in vitro selection, eluting bound RNAs with 10 μM tRNA^{Phe}. Aliquots of the collected fractions were loaded onto a 10% polyacrylamide gel containing 8 M urea for autoradiography.

Enzymatic probing

RNA transcripts were 5’-32P-labeled using [γ-32P]ATP and T4 polynucleotide kinase. RNAs were denatured at 70°C for 4 min and cooled to room temperature for 10 min in 0.25 M NaCl, 50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 2 mM spermidine, 0.2 mM EDTA. For the study of nuclease cleavage in presence of ligand, 6 μM tRNA was added. T1 cleavage was performed at 37°C for 30 s at a final concentration of 0.1 U/μl, V1 at room temperature for 5 min at 0.024 U/μl, S1 at 37°C for 1 min at 10 U/μl, including 1 mM ZnCl₂, in the reaction buffer. An equal volume of a gel loading buffer containing 9 M urea, 50 mM EDTA, pH 8, was added and the reaction products were separated immediately on 12% polyacrylamide gels containing 8 M urea.

Determination of $K_d$

RNA dissociation constants ($K_d$) for RNA ligands were determined by isocratic competitive affinity chromatography (Connell et al., 1993; Ciesiolk a et al., 1996). Approximately 10 pmol of internally labeled 32P-RNA was heated at 70°C for 4 min, brought to column buffer conditions + 3 μM ligand, and allowed to renature at 10 μM room temperature before loading onto a 500 μl column. Dissociation constants for ligand in solution were calculated from: $K_d = L \cdot (V_d - V_c) \cdot (V_e - V_c)$, where $L$ is the free ligand concentration used to isocratically elute RNA loaded onto the affinity column, $V_d$ is the median elution volume of RNA eluted in the continuous presence of free ligand (the isocratic elution volume), $V_e$ is the median elution volume measured in the absence of free ligand in the column buffer, and $V_c$ is the volume at which an RNA population having no interaction with the column would elute.

Fluorescence measurements

tRNA^{Phe} and aptamer RNAs were denatured separately in water at 1 μM for 4 min at 70°C, mixed, and cooled to room temperature for 10 min in column buffer before measuring the fluorescence on an LS50 Luminescence Spectrometer (Perkin-Elmer) by exciting the wybutine in native tRNA^{Phe} at 305 nm and monitoring emission between 340 and 550 nm.
References


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