Replacement of RNA hairpins by in vitro selected tetranucleotides

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ABSTRACT

An in vitro selection method based on the autolytic cleavage of yeast tRNA^{Phe} by Pb^{2+} was applied to obtain tRNA derivatives with the anticodon hairpin replaced by four single-stranded nucleotides. Based on the rates of the site-specific cleavage by Pb^{2+} and the presence of a specific UV-induced crosslink, certain tetranucleotide sequences allow proper folding of the rest of the tRNA molecule, whereas others do not. One such successful tetramer sequence was also used to replace the acceptor stem of yeast tRNA^{Phe} and the anticodon hairpin of E.coli tRNA^{Phe} without disrupting folding. These experiments suggest that certain tetramers may be able to replace structurally non-essential hairpins in any RNA.

INTRODUCTION

One of the most common RNA secondary structural element is the hairpin. RNA hairpins involved in tertiary interactions are in general phylogenetically conserved in both sizes and sequences. On the other hand, many hairpins are quite variable in sizes and/or sequences and are thus likely to be located on the surface of the molecule. For example, stem-loops (P4, P6, P7) located within the catalytic 'core' of the group I introns are highly conserved while others at the periphery of the 'core' (P2, P5, P8, P9) are not (1). Similar examples can be found in rRNAs (2). Substitution of phylogenetically non-conserved hairpins by short single-stranded loops are often of interest in the biochemical studies of RNA. A hairpin dispensable for the function is often non-essential for the folding of the RNA. The resulting RNA with a reduced size is more attractive for structure-function studies. There are numerous reports where non-conserved helices have been deleted or substituted with short loops with various success. Helices P9.1 and 9.2 of the Thermus thermophilus tRNA intron have been shown to be dispensable for the core catalytic activity (3) as well as helices P7.1 and 7.2 of the sunY intron (4). A simplified ribonuclease P RNA that excludes four non-essential helices of the E.coli M1 RNA has a catalytic efficiency similar to that of the native ribozyme in vitro (5). Helix II of the hammerhead ribozyme can also be substituted without abolishing its catalytic activity (6).

In an effort to understand how to successfully eliminate RNA hairpins which are not directly involved in tertiary folding, the anticodon hairpin of yeast tRNA^{Phe} was replaced by four single-stranded residues. We know from the tertiary structure of this tRNA (7,8) that the central core of tRNA^{Phe} is maintained by nine tertiary interactions involving the D and T stem-loops and the variable loop, whereas the anticodon hairpin and the acceptor stem are essential only for tRNA function. An in vitro selection method based on the specific cleavage reaction of tRNA^{Phe} by Pb^{2+} was used to isolate sequence variants of the tetranucleotide loop which gave optimal Pb^{2+} cleavage rates for derivatives lacking the anticodon hairpin of tRNA^{Phe}. Pb^{2+} cleavage of unmodified yeast tRNA^{Phe} at U17G18 has been studied extensively and the rate of this cleavage reaction correlate directly with the proper folding of tRNA^{Phe} (9–13). One ‘optimal’ sequence was also used to replace the anticodon hairpin of E.coli tRNA^{Phe} and the acceptor stem of yeast tRNA^{Phe}. The results of these experiments suggest that certain tetranucleotide sequences are capable of replacing structurally non-essential hairpins without affecting three dimensional folding.

MATERIALS AND METHODS

In vitro selection of circular RNA's that undergo autolytic cleavage with Pb^{2+} has been described in detail previously (14). After three rounds of selection, the resulting cDNA was cloned and individual variants were sequenced.

To allow immediate screening of individual variants for Pb^{2+} cleavage, minilysate DNA was amplified by PCR using one primer containing the T7 RNA polymerase promoter sequence and nucleotides 1 to 7 of yeast tRNA^{Phe} (5'TAATACGACTC- ACTATAACGGATT 3') and a second primer complementary to nucleotides 59 to 76 of yeast tRNA^{Phe} (5'TGTTGCG-AATTCGTGGA 3') to give an 80nt dsDNA. After transcription by T7 RNA polymerase (15) in the presence of [α-32P] CTP, the 63nt RNA was purified on a 15% polyacrylamide gel containing 7M urea. Recovered RNA was renatured at 85°C for 2 min in 15 mM MOPS pH 7.0 and incubated in 10 mM MgCl2 and 0.3 mM Pb(OAc)2 for 6 min at 22°C. The cleavage reactions were analyzed on 15% polyacrylamide, 7M urea gels.

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To precisely map the cleavage site of variants #4 and #8, cleavage of $^{32}$P labeled RNAs was compared to an alkaline hydrolysis ladder and a partial nuclease T1 digest of the same molecules. For all other variants the cleavage site was estimated from migration of cleavage products of [$\alpha$-$^{32}$P]-CTP labeled transcripts.

In order to determine Pb$^{2+}$ cleavage rates, RNA was synthesized by runoff transcription of BstNI digested plasmid DNA (15). Pb$^{2+}$ cleavage rates were measured at 1M RNA, 10 mM MgCl$_2$, 0.3 mM Pb(OAc)$_2$, 15 mM MOPS pH 7.0 at 25°C.

C60U mutations were introduced using one PCR primer complementary to nucleotides 47 to 76 of tRNA$^{Phe}$ (5'TGGTGGCAATTCTGTAATCGAACACAGGA 3') including the mutation (underlined) followed by transcription with T7 RNA polymerase.

The replacement of the anticodon hairpin of $E$.coli tRNA$^{Phe}$ (C3G, G71C and U60C) and the acceptor hairpin of "inverted" yeast tRNA$^{Phe}$ (Figure 2E) was performed using the DNA plasmids containing the wild type sequence and PCR amplification. In case of $E$.coli tRNA$^{Phe}$, a primer complementary to nucleotides 44 to 76 and 14 to 26 was used including the sequence A$^{26}$-UUAA-G$^{44}$, (5'TGGTGCGGGA-CTCGGATCGAAGGAACTATATGCTCTACC-GACT 3'). For the 'inverted' yeast tRNA$^{Phe}$ the primer was complementary to nucleotides 11 to 31 and 54 to 65 of yeast tRNA$^{Phe}$ and included the sequence G$^{65}$-UUAA-U$^{8}$, (5'TGGC-GCCGCTCTCCAACTGAGCTATTAACTGATCGATC- GACT 3'). The second primer used in both cases contained the T7 RNA polymerase promoter sequence from -17 to +1 (5'TAACGACTATAG 3'). RNA was generated by subsequent transcription with T7 RNA polymerase using DNA from PCR reactions.

RESULTS

To maximize the chance of obtaining tRNA derivatives which lack the anticodon hairpin but otherwise fold correctly, we replaced nucleotides 27 to 43 by a single stranded loop of four residues. Since four residues can span as much as 29 Å (16), they should be sufficient to bridge the 18.3 Å gap between the phosphates of the 'propeller-twisted' G$^\alpha$-A$^\delta$ base pair. Four residues were chosen instead of three or longer because the most common hairpin loops in 16S-like rRNAs (17) contain four residues. Thermodynamic studies also showed that four residue loops are more stable than hairpin loops of other sizes (18). The sequences of the four nucleotides were randomized (Figure 1) to allow selection of sequence variants that permit other potential interactions to occur in order to compensate for the loss of coaxial stacking of the D and anticodon helices.

The in vitro selection procedure described by Pan & Uhlenbeck (14) was used to isolate variants based on rapid self-cleavage with Pb$^{2+}$. Linear RNA transcripts were first circularized by T4 RNA ligase. Autolytic cleavage of circular RNA's with Pb$^{2+}$ generated linear molecules which were separated from the inactive circular RNA's on a denaturing gel. The cleaved RNA fraction was eluted from the gel, recircularized by the combined action of T4 polynucleotide kinase and T4 RNA ligase, reverse transcribed into DNA with AMV reverse transcriptase and amplified by PCR. After transcription with T7 RNA polymerase further cycles of selection and amplification were performed. Since no further significant enrichment of Pb$^{2+}$ cleavage activity was observed after two rounds of selection, the cDNA after the third round was cloned into the $E$.coli plasmid pUC18 and 33 colonies containing inserts were sequenced.

Twelve colonies had no alterations of the yeast tRNA$^{Phe}$ sequence other than at the randomized positions. The other 21 variants had at least one deletion or mutation at sites expected to be constant. Since we were interested in stem-loop replacement, only the 12 variants containing no other changes were tested for Pb$^{2+}$ cleavage (Table I). All variants could be cleaved specifically at the expected site between U17 and G18, although the efficiency of cleavage differed substantially. Four RNA variants were cleaved much less well than tRNA$^{Phe}$, suggesting that these RNA molecules are misfolded. Seven variants were cleaved quite efficiently under these conditions with three variants (#4, #8 and #15) being better than the others.

The variant containing the sequence UCUU (#1) showed an photocrosslink in tRNA$^{11}$ and the boxed nucleotides, C48 and U59, form a specific site. Cleavage at this site was as efficient as the background sequence of yeast tRNA$^{11}$, a G1A mutation and 4 randomized nucleotides between G26 and A44. The arrow indicates the Pb$^{2+}$ cleavage site of yeast tRNA$^{Phe}$ and the boxed nucleotides, C48 and U59, form a specific photocrosslink in tRNA$^{Phe}$.

Table 1. Sequences of selected variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>G$^{26}$-NNNN-A$^{44}$</th>
<th>Cleavage Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>UGAC</td>
<td>+++</td>
</tr>
<tr>
<td>8</td>
<td>UUAA</td>
<td>+++</td>
</tr>
<tr>
<td>15</td>
<td>CAAC</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>UCCU</td>
<td>++</td>
</tr>
<tr>
<td>9</td>
<td>UUUU</td>
<td>++</td>
</tr>
<tr>
<td>11</td>
<td>AAUC</td>
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</tr>
<tr>
<td>12</td>
<td>CCGA</td>
<td>++</td>
</tr>
<tr>
<td>13</td>
<td>ACCC</td>
<td>++</td>
</tr>
<tr>
<td>14</td>
<td>CUGC</td>
<td>+</td>
</tr>
</tbody>
</table>

* Conditions: 15mM MOPS, pH 7.0, 10mM MgCl$_2$, 0.3mM Pb(OAc)$_2$, 0.2μM RNA, 6 min at 22°C; +++: >15%; ++: 5–15%; +: <5% products observed.

Figure 1. Cloverleaf presentation of the circular RNA library; all variants contain the background sequence of yeast tRNA$^{Phe}$, a G1A mutation and 4 randomized nucleotides between G26 and A44. The arrow indicates the Pb$^{2+}$ cleavage site of yeast tRNA$^{Phe}$ and the boxed nucleotides, C48 and U59, form a specific photocrosslink in tRNA$^{Phe}$.
Two of the variants (4, 8) which cleaved efficiently were analyzed further to confirm that they fold like yeast tRNA^"Phe". The Pb^2+ cleavage rates under a variety of reaction conditions were derived from first-order kinetics of disappearance of substrate. The cleavage rates of both variants were essentially identical to yeast tRNA^"Phe" (Table II). At 10 mM Mg^2+, the cleavage rate increased proportionally with Pb^2+ concentration as was previously found with yeast tRNA^"Phe" (12). Finally, the rate of cleavage in 0.3 mM Pb^2+ as a function of varying Mg^2+ concentration from 2 to 70 mM showed a bell shaped curve with a maximal cleavage rate at about 10 mM (data not shown). This behavior is also very similar to the full length tRNA^"Phe" (12), suggesting that these molecules fold in a very similar fashion.

The Pb^2+ ion responsible for the cleavage reaction between U17 and G18 is directly coordinated by O' of U59 and N3 of C60 (10,11), as seen in the crystal structure of yeast tRNA^"Phe". When the C60U mutation is introduced in yeast tRNA^"Phe", cleavage rate is reduced by 12.5 fold (12). When C60U mutations were introduced in variants 4 and 8, cleavage rates were again reduced to a similar extent (Table II). Thus, Pb^2+ binding to variants 4 and 8 is likely to involve the same residues as in yeast tRNA^"Phe".

Another means to characterize the folding of yeast tRNA^"Phe" central core is to determine whether a cyclobutane dimer forms between nucleotides C48 and U59 upon irradiation with short wavelength UV-light (19). This crosslink is consistent with the crystal structure of tRNA^"Phe" since C48 in the variable loop and U59 in the T-loop are stacked on each other (5,6). Mutational studies show that the efficiency of this crosslink also depends on the correct tertiary structure of tRNA^"Phe" (19). When variants 4 and 8 were irradiated with shortwave UV-light for 6 min in 30 mM HEPES pH 7.4 and 10 mM Mg^2+, a slower migrating crosslinked species was rapidly formed. The crosslinking site was mapped as described previously (19), revealing the corresponding residue, C48, as the 5' crosslinking site (not shown). The efficient crosslinking of both variants provides additional evidence that the central core is folded correctly.

Since the selection experiment was performed in the background sequence of yeast tRNA^"Phe", it is of interest whether the same tetranucleotide sequences can be used to replace the anticodon hairpin of another tRNA. We choose a derivative of E.coli tRNA^"Phe" (Figure 2C) which can also cleave with Pb^2+ between residues C17 and G18 (12) at a rate similar to that of yeast tRNA^"Phe" (Table II). Although there is no crystal structure for this tRNA, numerous biochemical and structural characterizations indicate that E.coli tRNA^"Phe" is structurally very similar to yeast tRNA^"Phe" (12,20–22). This tRNA differs from yeast tRNA^"Phe" at 28 nucleotide positions but it shares 16 out of

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Table II. Pb^2+ cleavage rates of various constructs

<table>
<thead>
<tr>
<th>tRNA</th>
<th>$k_{obs} (x10^3 s^{-1})$</th>
<th>$k_{rel}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast tRNA^&quot;Phe&quot;</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Variant 8 (C-UUAA-A^4)</td>
<td>1.4</td>
<td>0.93</td>
</tr>
<tr>
<td>Variant 8 (C-UUAA-A^4), C60U</td>
<td>&lt;0.1</td>
<td>&lt;0.07</td>
</tr>
<tr>
<td>Variant 4 (C-UUAA-A^4)</td>
<td>1.4</td>
<td>0.93</td>
</tr>
<tr>
<td>Variant 4 (C-UUAA-A^4), C60U</td>
<td>&lt;0.1</td>
<td>&lt;0.07</td>
</tr>
<tr>
<td>E.coli tRNA^&quot;Phe&quot;</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>E.coli tRNA^&quot;Phe&quot; (A-UUAA-G^4)§</td>
<td>0.9</td>
<td>0.55</td>
</tr>
<tr>
<td>&quot;inverted&quot; Yeast tRNA^&quot;Phe&quot;</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>&quot;inverted&quot; Yeast tRNA^&quot;Phe&quot; (G-UUAA-U^B)</td>
<td>1.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

a All reactions were performed at 1 mM RNA, 10 mM MgCl₂, 0.3 mM Pb(OAc)₂, 15 mM MOPS pH 7.0 and 25°C.

b Relative to the corresponding full-length RNA; §: also include C3G, G71C and U60C mutations.
19 nucleotide positions that are involved in tertiary interactions (Figure 2C). However, the two tertiary interactions adjacent to the anticodon hairpin in yeast tRNA^Phe^ (G26-A44, G10-C25-G45) are different in E.coli tRNA^Phe^ (A26-G44, G10-C25-U45). Since neighbor effects are often important in RNA folding, it is unclear whether the anticodon hairpin could be replaced by the same tetramer. Replacement of the anticodon stem of E.coli tRNA^Phe^ with the UUAA sequence of variant #8 (Figure 2D) decreased the rate of Pb^{2+} cleavage by less than 2 fold (Table II). Thus, the same tetranucleotide sequence can be used to delete the anticodon hairpin in two tRNAs with different adjacent sequences.

When irradiated with UV-light, E.coli tRNA^Phe^ forms multiple crosslinked species. This is thought to be due to the four adjacent G-C base pairs in the anticodon stem (22). Since our construct does not contain the anticodon stem, we expect to obtain only one crosslinked RNA species after irradiation with UV-light. This was indeed observed (not shown), supporting the view that the four G-C base pairs are the primary cause of forming alternative conformers in native E.coli tRNA^Phe^. This result also suggests that structural heterogeneity could be circumvented by replacing hairpins that are potentially prone to form alternate structures.

The ability of the sequence UUAA to replace two anticodon hairpins in different sequence context raises the possibility that it can be used for the replacement of other RNA hairpins. We first constructed a circularly permuted tRNA (denoted ‘inverted’ tRNA^Phe^) where the 5' and 3' ends were located in the anticodon stem and a 6 base pair hairpin with a stable GA3 loop replaced the acceptor stem (Figure 2E). This construct also cleaved with Pb^{2+} between U17 and G18 in the D-loop with a rate similar to that of yeast tRNA^Phe^ (Table II) confirming that the anticodon loop is dispensable.

The ‘acceptor hairpin’ of the ‘inverted’ tRNA^Phe^ is not expected to be involved in tertiary interactions and should therefore be interchangeable with a tetranucleotide loop as for the anticodon hairpin. When nucleotides A66 to U7 were replaced with the sequence 5' UUAA 3' (Figure 2F), the resulting molecule cleaved with Pb^{2+} at the same rate compared to the full length ‘inverted’ tRNA (Table II). Additionally we were able to induce an UV-crosslink into this molecule at a similar efficiency as for the full length RNA. Both experiments suggest that the acceptor stem of yeast tRNA^Phe^ can be successfully substituted by the same tetranucleotide sequence as the anticodon hairpin and this RNA containing 54 nucleotides is also sufficient for formation of the correct tRNA central core.

**DISCUSSION**

We have shown that it is possible to replace structurally non-essential stem-loops with single stranded loops of four nucleotides. It is clear from this work that the sequence of the tetranucleotide determines whether the resulting RNA molecule has proper structure. To avoid testing of all possible combinations individually, *in vitro* selection was used to isolate the optimal sequence variants. The same selection protocol can be used to replace non-essential hairpins in other RNA's that undergo autolytic cleavage.

Although three rounds of selection were performed, the resulting sequence variants still showed a wide range of cleavage efficiency. This might be due to non-optimal cleavage conditions used in the selection procedure. As discussed previously (14), Pb^{2+} cleavage is not the only criteria for selection, the usage of protein enzymes introduces additional selection pressure. It is plausible that variation in the tetranucleotide sequences may affect the efficiencies of T4 polynucleotide kinase and T4 RNA ligase to act at the cleavage site. This selective pressure could have been avoided by the use of a second PCR primer comprised of the T7 promotor sequence and nucleotides 1 to 26 of tRNA^Phe^. Thus it should be possible to select and amplify variants of this library which are cleaved at the expected site without the circularization steps.

Nevertheless, the best sequence variants showed cleavage rates and specificity almost indistinguishable from that of the yeast tRNA^Phe^. By all experimental criteria we have carried out, they possessed tRNA central folds likely to be identical to that of yeast tRNA^Phe^. Due to the limited number of variants it is not possible to derive a consensus sequence of folding permissive sequences (if any exists). Since only a dozen variants were tested, we believe that other tetranucleotide sequences suitable for this purpose are surely to exist.

It is not obvious why some sequences work far better than others. One possible explanation is that permissive sequences promote intermolecular base pairing. This would imply concentration dependent cleavage rates which was not observed experimentally (data not shown). Another possibility is that these sequences are least likely to form alternate conformers. This is again unlikely since in two cases identical sequences can be used to replace other hairpins in different tRNA's (Table II, Figures 2,3). The successful application of using the same permissive tetranucleotide loops to replace RNA hairpins in different molecules raises the possibility that certain tetranucleotide loops that can be used for stem-loop replacement in any or most RNAs.

We have rationalized earlier why tetranucleotides were chosen to replace RNA hairpins. Obviously, loops of other sizes could also serve for this purpose. To maximize the effect of size reduction, a selection using trimers of randomized sequences
might be worth investigating. Although three nucleotides can bridge a maximum distance of about 21 Å (16), the conformational arrangement of the loop nucleotides is probably less flexible compared to tetramers. We therefore would expect that the number of permissive trimers is very limited and more sequence specific. Indeed, a previous attempt to replace the acceptor hairpin of the ‘inverted’ tRNAPhe by a three nucleotide loop (G65-AUU-U8) resulted in a molecule that did not cleave with Pb2+ at all (T.P., T.D. & O.C.U. unpublished results).

Phylogenetic comparison of proposed secondary structures for 16 S-like ribosomal RNAs (17), the 23 S ribosomal RNAs (2) and SRP-RNAs (24) revealed several natural examples where RNA hairpins may have been replaced by tetranucleotides. This might be explained by a change or loss of specific functional interactions of the RNA due to evolutionary pressure which made the hairpins dispensable. For example, the [(184–186)–(191–193)] helix in 16 S-like ribosomal RNA within eubacteria, archaeabacteria and chloroplasts has been replaced in plant mitochondria by the tetramer 5' AAAA 3'. The hairpin (1722–1735) in the 5' half of 23S ribosomal RNA of Desulfurococcus mobilis has been replaced by the tetramer 5' GAAA 3' in Halobacterium halobium, where both organisms are archaeabacteria. Another example is a seven nucleotide hairpin (169–175) within the SRP-RNA of Canis species (eucaryote) which is substituted in Halobacterium halobium by the sequence 5' UUCG 3'. This might actually be a case where a hairpin has been inserted instead of the tetramer since this hairpin occurs exclusively in eucaryotes. These naturally occurring examples of hairpin replacement by tetramers in completely different contexts clearly show that 6-nucleotide loops may be used as a tool to isolate RNA variants with novel structural features. Successful replacement of structurally isomorphic elements may help to assess folding information for other (larger) RNA molecules where the tertiary structure is otherwise hardly accessible. Furthermore, size reduction is highly desirable for structural investigation of RNA by NMR or X-ray crystallography.

This work confirms once more that in vitro selection can be used as a tool to isolate RNA variants with novel structural features. Substitution of the anticodon hairpin of yeast tRNAPhe by tetranucleotides of randomized sequences leads to the result that distinct sequences are required to maintain tertiary structure. Since one optimal sequence can be used to replace two other RNA hairpins, it would be interesting to know how general these ‘optimum-sequences’ can be applied and to see whether these sequences are compatible with RNAs other than tRNAs.

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