Novel RNA Substrates for the Ribozyme from *Bacillus subtilis* Ribonuclease P Identified by *in Vitro* Selection†

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ABSTRACT: Novel RNA substrates for the ribozyme from *Bacillus subtilis* ribonuclease P (P RNA) have been obtained by *in vitro* selection. The selection method involves cleavage of a circular RNA library by the P RNA, isolation of the linear cleavage product, and regeneration of circular RNA to allow amplification and multiple cycles of selection. The use of circular RNA ensures that potential substrates can be selected without restricted location of the cleavage site. Such a selection method has been used previously to isolate RNA motifs that undergo autolytic cleavage with Pb^{2+} [Pan, T., & Uhlenbeck, O. (1992) *Biochemistry 31*, 3887−3895]. The circular RNA pool after eight cycles of selection was cleaved by the *B. subtilis* P RNA as efficiently as a pre-tRNA^{Phe} substrate, estimated to be more than 10 orders of magnitude better than the unselected RNA library. Kinetic analysis of individual variants showed that the k_{cat}/K_M of the selected RNA was up to 4-fold higher than that of the pre-tRNA^{Phe}. When cleavage was carried out with *Escherichia coli* P RNA, the selected RNA was 10−60-fold less reactive than the reaction of the pre-tRNA^{Phe}. Two distinct classes of variants are selected, both of which appear to differ significantly from the known P RNA substrates. Terminal truncation experiments suggest that a large number of nucleotides in the class I variants can be deleted without affecting the cleavage activity. The resulting minimal class I substrates contain a short stem−loop with no other apparent helical structures. The class II substrates are cleaved within a putative helical stem that is formed entirely by the primer sequences. These results indicate that catalysis by eubacterial P RNAs is far more versatile than previously thought and raise the possibility that RNase P may process other yet to be defined RNAs *in vivo*.

The substrate specificity of a catalytic RNA is often defined by RNA structures that are formed between the ribozyme and the substrate. For most ribozymes, substrate recognition occurs primarily through Watson−Crick base pairing between a region on the ribozyme and a region on the substrate. For example, the *Tetrahymena* group I ribozyme employs a hexanucleotide sequence, 5′-GGAGGG, also termed the internal guide sequence, that base pairs with six nucleotides immediately 5′ to the cleavage site (Cech, 1990; Strobel & Cech, 1993). One exception to this mode of recognition is the ribozyme from eubacterial ribonuclease P (P RNA). P RNA catalyzes a single cleavage reaction in the tRNA precursor to produce the mature 5′ end of all tRNAs (Altman et al., 1993; Pace & Smith, 1990). In this case, substrate binding occurs through still poorly defined tertiary interactions between the folded tRNA structure and the P RNA (Harris et al., 1994; Westhof & Altman, 1994). Regions in tRNA that interact with P RNA consist of the coaxial stacked acceptor stem and the T stem, the conserved nucleotides in the T loop, and the 3′-CCA.

If specific binding to an RNA structure is the key for P RNA catalysis, then any RNA structure that has the potential to fit into the binding surface of P RNA may become a substrate. Such a hypothesis is supported in part by the findings that smaller RNA molecules originating from the tRNA precursor are indeed efficient substrates for P RNA (McClain et al., 1987; Perreault & Altman, 1992; Schlegl et al., 1992). For example, the acceptor stem and the T stem in tRNA can be covalently linked to generate a P RNA substrate with a contiguous helix of 12 base pairs, the T loop, and the 3′-CCA. A recent *in vitro* selection experiment identified *Escherichia coli* P RNA substrates that contain a structure analogous to the coaxial stacked acceptor stem and the T stem, with a hairpin loop resembling, in part, the T loop in tRNA (Liu & Altman, 1994). Interestingly, the important 3′-CCA nucleotides in pre-tRNA are absent from these selected substrates, indicating that the substrates may adopt alternate ways to fit into the binding site of *E. coli* P RNA.

This paper explores other possible RNA structures that can be recognized and specifically cleaved by the *Bacillus subtilis* P RNA. An *in vitro* selection protocol is employed in which P RNA substrates are selected without prior knowledge of the location of the cleavage site. This is achieved by cleaving the circular form of the RNA library, followed by enzymatic ligation at the cleavage sites (Pan & Uhlenbeck, 1992a,b). Two classes of novel RNA substrates were identified that can be cleaved by the *B. subtilis* P RNA, with efficiencies (k_{cat}/K_M) as much as 4-fold higher than that of a pre-tRNA^{Phe} substrate. Further biochemical characterizations show that these new substrates contain structures that are distinct from pre-tRNAs and the other known P RNA substrates.

MATERIALS AND METHODS

Preparation of the RNA Library. The double-stranded DNA template was made by annealing and extending 100

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pmol of two synthetic DNA oligonucleotides with 375 units of MuLV reverse transcriptase at 37 °C for 25 min. The DNA oligos, 5′-TGGTGCGAATTCTGAA(N)$_{35}$GATCTT-CAGTCTGG and 5′-TAATACGACTCACTATAACGG-ATTCGCTCAA(N)$_{35}$CCAGACTGAAGATC, contain a complementary 14-nucleotide sequence (underlined) and a total of 70 randomized nucleotides. To generate large quantities of DNA for transcription, 1.2 mL of PCR reaction was carried out using the primers 5′-TGGTGCGAATTCT-GAA and 5′-TAATACGACTCACTATAACGGATTCGCT-CAA with the double-stranded DNA under the following conditions: 95 °C, 50 s; 37 °C, 50 s; 72 °C, 2 min; 5 cycles. Transcription by T7 RNA polymerase was performed as described previously (Milligan et al., 1987; Pan & Unlenbeck, 1992a) to produce 1.2 nmol of RNA, more than 80% of which contains a 5′-monophosphate. By assuming that 20 identical RNA molecules are present for each sequence, the library contains ~3 × 10^{13} RNA variants.

In order to synthesize the circular RNA library, ~4 μM linear RNA was incubated in 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 12 μM ATP, 15% DMSO, and 1 unit/μL T4 RNA ligase (New England BioLabs) at 37 °C for 2 h. The circular RNA was then purified from linear RNA on 10% polyacrylamide gels containing 7 M urea. The yield of circular RNA was about 40% after gel purification.

The Selection Procedure. The purified circular RNA was renatured by heating at 85 °C for 2 min in 50 mM Tris (pH 8.1), cooled to ambient temperature, and incubated with 10 or 25 mM $MgCl_2$ for 5 min at 22 °C and 5 min at 37 °C. The *B. subtilis* P RNA was renatured as described (Pan & Zhong, 1994; Pan, 1995). An equal volume of the circular RNA library and P RNA was then mixed to initiate the reaction at 37 °C. Aliquots were taken at timed intervals up to 30 min, and the cleavage reaction was stopped by the addition of 1.2 vol of 9 M urea/25 mM EDTA. The reaction mixture was separated on denaturing polyacrylamide gels, and the products were quantitated using a phosphorimager (Fuji Medicals). For this RNA library, the separation of circular and linear molecules was extremely efficient. The linear RNA products were then eluted from the gel, ethanol-precipitated, and redissolved in water. To disrupt potential RNA structures at the religation site, the linear RNA was heated at 90 °C for 1 min, followed by quick cooling on ice. Ligation using T4 RNA ligase was then carried out under the conditions described earlier at 16 °C for 1.5 h and at 37 °C for 30 min. The mixture was extracted with an equal volume of phenol/chloroform and precipitated with ethanol. Reverse transcription was performed in 20 μL of 20 mM Tris (pH 8.3), 40 mM KCl, 0.2 mM dNTPs, 1 μM primer, and 3 units of AMV reverse transcriptase at 42 °C for 10 min. PCR amplification was carried out under the following conditions: 94 °C, 50 s; 45 °C, 50 s; 72 °C, 1 min; 18−24 cycles. The PCR mixture was ethanol-precipitated and used for transcription without further purification.

Characterization of the Selected RNA Variants. After eight cycles of selection, the cDNA was PCR-amplified, purified, cloned, and sequenced as described in Pan and Unhlenbeck (1992a). To test the individual variants for cleavage by P RNA, α-^{32}P-labeled RNA was obtained from transcription by using PCR-amplified templates from plasmid DNAs. Cleavage reactions were carried out under single-turnover conditions with 50 nM *B. subtilis* P RNA at either 10 or 25 mM $MgCl_2$ and 50 mM Tris (pH 8.1) at 37 °C. For the class II variants, no cleavage product was observed initially with linear, α-^{32}P-labeled RNA. These variants were then circularized using T4 RNA ligase and retested for cleavage. k_{cat} and K_M values for several variants were also determined under multiple-turnover conditions as described in Pan and Zhong (1994).

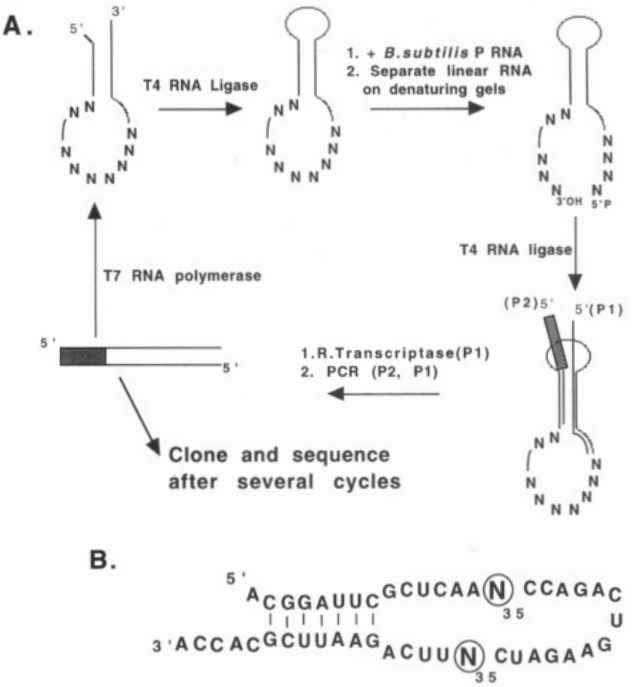

FIGURE 1: (A) Experimental strategy for selecting novel P RNA substrates. The randomized regions in the RNA library are shown as N and the primers are depicted as lines. Only one cleavage site is shown in the diagram, although cleavage at any site will generate a linear RNA product of equal length. P1 and P2 represent the DNA primers. The filled box of P2 corresponds to the promoter sequence of T7 RNA polymerase. (B) Sequence of the RNA library with a total of 70 randomized nucleotides.

To determine the minimal lengths of RNAs that remain efficient substrates for *B. subtilis* P RNA, RNA variants were either 5′-^{32}P-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase or 3′-^{32}P-labeled using [5′-^{32}P]pCp and T4 RNA ligase (England et al., 1980). Partial alkaline hydrolysis of end-labeled RNA was performed in 1 mM glycine and 0.4 mM $MgSO_4$ (pH 9.5) by boiling for 1 min. The hydrolyzed RNA mixture was then neutralized by the addition of Tris (pH 8.1) to 50 mM, renatured, and cleaved as described earlier. The reaction mixture was analyzed on 8% denaturing polyacrylamide gels.

RESULTS

Selection of Novel P RNA Substrates. In order to search for RNA structural motifs that are potential P RNA substrates, a selection protocol is applied in which RNA variants can be isolated regardless of the location of the cleavage site (Figure 1A). Such a selection procedure was developed previously to obtain RNA variants that undergo autolytic cleavage with Pb^{2+} (Pan & Uhlenbeck, 1992a). The method involves preparation of the random RNA library in its linear form, followed by circularization with T4 RNA ligase. Incubation of the circular RNA with *B. subtilis* P RNA generates products that are linear. The linear products can then be separated from unreactive circular RNAs on denatur-

ing gels. In order to amplify the RNA products for the next cycle of selection, the products are recircularized using T4 RNA ligase. The resulting circular RNAs can now be converted into DNA templates by reverse transcription and PCR to allow multiple cycles of selection.

The RNA library contains a total of 70 randomized nucleotides separated in two segments of 35 nucleotides each by a constant region of 14 residues (Figure 1B). These 14 nucleotides are designed such that the DNA template of the entire library can be synthesized in two oligonucleotides to improve yield (Bartel & Szostak, 1993). With 70 randomized nucleotides, potential substrates with the size of a minimal P RNA substrate (~35), as well as those approaching a complete transfer RNA, can be selected.

Cleavage efficiency of pre-tRNA substrates by P RNA exhibits strong dependence on the concentrations of Mg^{2+} and monovalent ions such as K^+ (Reich et al., 1988; Smith & Pace, 1993; Beebe & Fierke, 1994). At 25 mM $MgCl_2$ and 1 M KCl at pH 8, *B. subtilis* P RNA cleaves a pre-tRNA^{Phe} substrate with a k_{cat}/K_M of $\sim 3 \times 10^7$ M^{-1} min^{-1}. At 25 mM $MgCl_2$ alone, the k_{cat}/K_M is reduced by ~10-fold ($\sim 3 \times 10^6$ M^{-1} min^{-1}). k_{cat}/K_M of this pre-tRNA^{Phe} substrate is further reduced by ~15-fold at 10 mM Mg^{2+} ($\sim 2 \times 10^5$ M^{-1} min^{-1}). Folding studies of the *B. subtilis* P RNA and the pre-tRNA^{Phe} substrate suggest that both RNAs are completely folded at 10 mM $MgCl_2$ alone (Pan, 1995). Thus, it is unclear whether the requirement of high concentrations of K^+ and Mg^{2+} is due to the intrinsic nature of catalysis by P RNA or to specific interactions between the P RNA and the pre-tRNA. This was considered by performing *in vitro* selection at suboptimal cleavage conditions for pre-tRNA substrates: only 25 mM Mg^{2+} was included in the first four rounds of selection, followed by four additional rounds at 10 mM Mg^{2+}. Selection was also carried out at the 1 μM RNA library over 0.2 μM P RNA for the first six cycles, followed by 0.04 μM P RNA at rounds 7 and 8. The reductions in Mg^{2+} and ribozyme concentrations at later cycles should further facilitate the accumulation of more active substrates.

The enrichment of P RNA substrates in the selection experiment is shown in Figure 2. A substantial increase in cleavage rate was apparent at round 4. In round 5, Mg^{2+} concentration was decreased from 25 to 10 mM, which resulted in a slight drop in the cleavage rate. In subsequent cycles the cleavage rate increased progressively. At round 8, the RNA library was cleaved at ~0.2 min^{-1}, which is better than the cleavage of pre-tRNA^{Phe} under identical conditions (0.15 min^{-1}). Since further selection may decrease the chance of finding many different classes of substrates, the cDNAs from round 8 were cloned, and 22 clones were sequenced. Fourteen independent sequences were found among these 22 clones (Table 1). These variants can be initially assigned into two different groups on the basis of the conservation of a hexanucleotide sequence in one group, UAANCA (underlined in Table 1).

Characterization of Selected Variants. Cleavage reactions were carried out with a trace amount of ^{32}P-labeled RNA in the presence of 50 nM P RNA. This concentration of P RNA was at least 4 times below the K_M values of selected variants. Therefore, the reactivity of these variants, corresponding to the cleavage rate divided by the concentration of P RNA, was interpreted as k_{cat}/K_M. For the group of variants containing the conserved UAANCA sequence, rapid cleavage

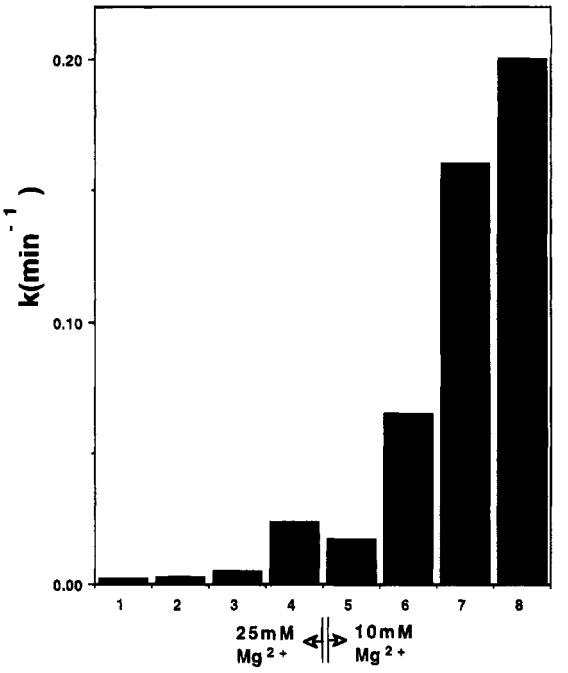

FIGURE 2: Accumulation of P RNA substrates in the selected RNA pools. Rounds 1–4 were carried out with 25 mM $MgCl_2$, and rounds 5–8 were performed in 10 mM $MgCl_2$.

at a specific site was observed. These variants are designated class I substrates. The remaining five variants are designated class II substrates; they were initially tested in their circular forms. The relative reactivities of individual variants compared to a pre-tRNA^{Phe} substrate are shown in Table 1. All 14 variants showed reactivities ranging from 0.2–3.6-fold to that of the pre-tRNA^{Phe}. For all variants tested, the relative reactivity remained constant for cleavage reactions carried out at 10 or 25 mM Mg^{2+}, although the absolute k_{cat}/K_M values under these conditions differed by about 15–20-fold. Steady-state kinetic parameters of two class I (10, 22) and one class II (17) variants revealed 3–20-fold lower K_M values for these variants at 25 mM Mg^{2+} (Figure 3). By assuming that, like pre-tRNAs (Smith & Pace, 1993), the K_M's of these variants also reflect substrate binding, then both class I and class II substrates appear to bind to P RNA at higher affinities than pre-tRNA (Figure 3).

Not only are these variants active substrates for *B. subtilis* P RNA, they are also cleaved specifically by *E. coli* P RNA. This is not surprising considering that both P RNAs contain identical active sites. Compared to pre-tRNA, however, these variants are 10–60-fold (relative k_{cat}/K_M) more selective in cleavage by *B. subtilis* P RNA (Table 1). Since these two P RNAs differ significantly in their secondary structural motifs (Haas et al., 1991; Pan, 1995), this result suggests that the selected substrates may contact unique regions in the *B. subtilis* P RNA that are absent from the E. coli P RNA.

Location of the cleavage site for the class I substrates is mapped in the 3′ randomized region (Table 1, Figure 3). In all cases, cleavage occurs 5′ to a G residue. Interestingly, the conserved hexanucleotide in the randomized region, UAANCA, is always located 11 or 12 residues 3′ to the cleavage site. In addition, several nucleotides immediately 5′ and 3′ to this conserved sequence are selected to base pair with each other. These base pairs form the helical stem of a hairpin loop containing this hexanucleotide sequence (Figure 3). Such a hairpin loop is reminiscent of the T

Table 1: Sequence and Reactivity of Selected P RNA Substrates

Variant[a]	Sequence[b]	Reactivity[c]
Class I substrates:		
#22 (3)	GUUAAGUGAGGACGUGACAGUAGACGUCCGUUC; CACACACCAAUCACCGAAAACUAAGGUAAUCACCC	3.6 (12)
#13	GUCCCAGGGAUACCCGCUUUAUGAGCAAGGGUGAC; CAAUCAUGUAAUUAGAGACUAACCAUGUCG	3.1 (19)
#10	AGGUCUGGUUGAGAGGAUGCUCAGAUACCAACCG; CAACACCAUACCGUAACUUGAAGUAAACAUCC	1.6 (23)
#20	UCUAGGAUAGAACGCCAAUACAAAUGGCAAUAUUC; CUGCAUACCAAUCGAAUUACCAAGUAACCAGAC	0.9 (10)
#3	GGAAAGGUCGUUAUUACUGUAGAGUACUUAGCGAU; ACCGGAAAACACUCGGAUUACGAACCUAACCAUGA	0.7 (18)
#14	GAAGGCAAUCGGAGAUGAGUAUCUCACACAGGAUG; CCAAACUCAAUGUAUAUUCCGACUAACCAUGG	0.6 (15)
#2 (2)	CACUGGCAGGUCGUAUAGACAGCUAUGCGCAUGUG; CCAUACAACACUUCGAAUGUUCAAGCUAAGCAUGC	0.5 (9)
#16	CACUGGCAGGUCGUAUAGACAGCUAUCGCGAUGUG; CCAUACAACACUUCGAAUGUUCAGCUAAGCAUGC	[0.3]
#1	UUGAAGUCAGGGGCCUCCCAGGAACUUCCCUGAC; CAAAUAACUCAUAACGAUUACUAGAGUAACCACC	0.2
Class II substrates[d]:		
#17	GUACGUGCGUAGGGUGGAGCAGACGGAUUACCCCA; CUACCAUGUAGACCGGAGCGAUGAAGUGCAAACC	[2.4]
#4 (5)	GGAUCAGAGCGCUGUUAGUGGGUUCUCGUUGGAUC; GACCACUACCCACUAGCUCAGAAGGUACUAUAC	1.8 (60)
#7 (2)	CGUGUUUGUGGAGACAUGUUGAUAGCCCCUAGUAA; CGACCUCCAUAGAGUCCUGCAAAGGUGAUGAGCCC	1.4
#21	GGCCACAAAGGGCUUUGGGAGCGCACUGCAGGAC; CGACUCCAAACGAACAUGGAUUAGUUCACACAA	[0.8]
#8	GUCUGGGGGAUGCCGAUCCCGUAAGUUGUGGCCCU; CACCAGUUCUGACAUAUUUGGAGCAAUGCAAUAC	[0.6]

[a] The numbers in parentheses show the total number of identical clones from 22 sequences. [b] The sequences of the 5′ and 3′ randomized regions are separated by a semicolon. For the class I substrates, the cleavage site is located between the highlighted residues. Conserved nucleotides among the class I substrates are underlined. Nucleotides that are presumably involved in forming Watson–Crick base pairs are shown in italics. [c] k_{cat}/K_M of selected substrates divided by k_{cat}/K_M of the pre-tRNA^{Phe} at 10 mM $MgCl_2$ (0.2×10^6 M^{-1} min^{-1}). The numbers in parentheses are values of k_{cat}/K_M with *B. subtilis* P RNA divided by k_{cat}/K_M with *E. coli* P RNA (pre-tRNA^{Phe} substrate = 1). The values in brackets are reactivities determined at 25 mM $MgCl_2$. [d] Circular molecules only.

stem–loop in pre-tRNA or in selected *E. coli* P RNA substrates (Liu & Altman, 1994). However, the loop sequence, UAANCAY, substantially differs from that of the T loop, UUCRANY, and those of the selected *E. coli* P RNA substrates, (G/U)UCACCC or CUANY(U/A)C. Importantly, the acceptor stem-like helix generally found in all other P RNA substrates is absent from the class I variants.

Cleavage of a partially hydrolyzed mixture of ^{32}P-end-labeled RNA was carried out to determine the minimal size of class I substrates (Forster & Symon, 1987; Pan & Uhlenbeck, 1992a,b). If the hydrolyzed RNA fragments in the mixture remain active, they will be cleaved by the ribozyme and thus diminish in intensity with respect to the same fragments in the mixture not treated with P RNA. For variants 10 and 22, almost all nucleotides 5′ to the cleavage site can be deleted without loss of activity (Figure 4). On the other hand, only four nucleotides from the 3′ end can be deleted. Thus, it appears that the determinants of class I variants are located 3′ to the cleavage site. This characteristic is similar to that of the standard pre-tRNA substrate in which the determinants for P RNA reaction are also located 3′ to the cleavage site (Smith & Pace, 1993; Harris et al., 1994). Unlike pre-tRNAs, the 3′-CCA nucleotides are not required for efficient cleavage of class I substrates (Figures 3 and 4).

The cleavage site for the circular class II substrates was determined in two ways. First, the linear product was isolated and ^{32}P-labeled at the 5′ end; partial hydrolysis and nuclease T1 digestion patterns of the labeled product revealed that the cleavage site is located in the 5′ primer region (not shown). Second, the precise location of the cleavage site was mapped by run-off reverse transcription using the 3′ primer (Figure 3). This cleavage site is only two nucleotides removed from the 5′ end of the linear RNA transcript. When the class II variants were ^{32}P-labeled at the 5′ end, the cleavage activity (k_{cat}/K_M) of these variants was found to be identical to that of the circular form (Figure 3). Unlike the class I substrates, no conserved sequences are known among the class II variants.

DISCUSSION

Novel RNA substrates for the ribozyme from *B. subtilis* RNase P have been identified by *in vitro* selection. Most ribozymes recognize their substrates through intermolecular Watson–Crick base pairs. In contrast, P RNA recognizes an RNA structure through tertiary interactions. Since the binding surface of P RNA seems to be quite large (Harris et al., 1994; Westhof & Altman, 1994), RNA motifs other than pre-tRNA could, in principle, fit into the binding site to orient a phosphodiester bond into the active site of P RNA. By using a selection procedure that randomizes the site of cleavage, a large number of structural motifs can be explored.

The isolation of two distinct classes of novel P RNA substrates is testimonial to the versatility of P RNA in cleaving a variety of RNA motifs. The previously known P

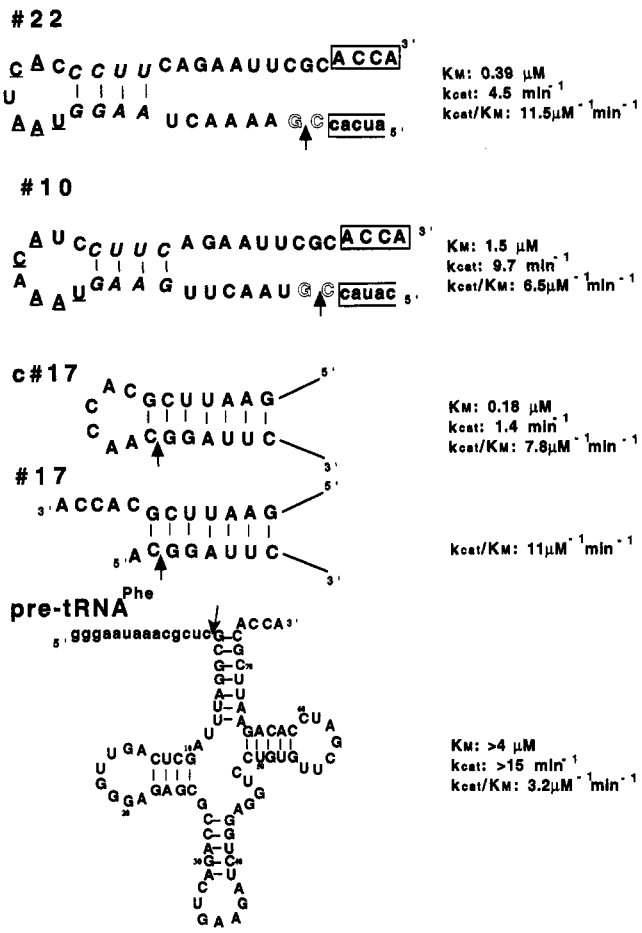

FIGURE 3: Partial sequences and putative secondary structures of some selected variants. For variants 10 and 22, all but one of the nucleotides 5′ to the cleavage site can be deleted in the terminal truncation experiment. The four 3′ most nucleotides that can be deleted are boxed. For variant 17, only the part of the primer sequence is shown that may form a helical stem. The cleavage sites of variants 10 and 22 are indicated by arrows.

RNA substrates can be thought of as modified versions of the structure adapted by the acceptor stem and the T stem−loop of tRNA. This motif consists of two coaxial stacked helices with a total of 11 or 12 base pairs, the unpaired nucleotides in the T loop, and the 3′-CCA (McClain et al., 1987; Liu & Altman, 1994). Superficially, the class I variants share some common features with the tRNA substrates. For example, these variants contain a hairpin structure with conserved loop residues located 11 or 12 nucleotides 3′ to the cleavage site. This hairpin structure is reminiscent of the T stem−loop in pre-tRNA, although the loop sequences differ significantly. It is conceivable that this hairpin may fit into a similar stem−loop binding stie on the *B. subtilis* P RNA. The detailed interactions at this binding site may vary depending on the actual loop sequence.

Perhaps the most striking difference between the class I variants and the pre-tRNA is that they lack an "acceptor" stem-like structure despite the presence of similar numbers of residues (Figure 3). There are two possible explanations. (1) The A,U richness in this "nonhelical" region promotes the conformational flexibility of these residues, so that a helical structure can be formed upon binding to P RNA. (2) Many residues in this region originate from the 3′ primer and hence are invariant. These residues may participate directly in interactions with the ribozyme that are not present in the pre-tRNA−P RNA complex. Mutagenic studies of these residues are now in progress to test this hypothesis.

One other significant difference between the class I variants and pre-tRNAs is the requirement of the 3′-CCA nucleotides. Although CCA is part of the 3′ primer in the selected substrates, they can be deleted without affecting the cleavage efficiency (Figure 4). The structural motif that interacts directly with the CCA nucleotides of the pre-tRNA has been mapped (LaGrandeur et al., 1994; Oh & Pace, 1994; Kirsebom & Svard, 1994), and the presence of CCA contributes to 2.0−2.6 kcal in binding affinity (Oh & Pace, 1994). It appears that class I substrates no longer require the contribution of this interaction.

The proximity of the cleavage site to the 5′ end of the linear class II variants implies that all determinants for P RNA recognition are located 3′ to the cleavage site for class II variants. The location of the cleavage site also results in selective advantages for these variants, since they can be amplified without the need for recircularization. It is interesting to note that the circular forms of these variants are almost equally as active as the linear forms. By ligating the 5′ and 3′ ends of the pre-tRNA^{Phe} used in this study (Figure 3), a circular pre-tRNA can be made with an acceptor hairpin containing 18 nucleotides in the loop. This circular pre-tRNA^{Phe} substrate is cleaved by *B. subtilis* P RNA with a k_{cat}/K_M reduced by ~8-fold compared to the linear pre-tRNA^{Phe} (T. Pan, unpublished results). Circularly permuted pre-tRNAs with an acceptor hairpin of more than 11 nucleotides could also be cleaved by *E. coli* P RNA, with k_{cat}/K_M reduced by 8−14-fold (Nolan et al., 1993). Compared to these circular pre-tRNAs, the class II variants have much higher k_{cat}/K_M values for the *B. subtilis* P RNA.

Although the selection protocol used here is a straightforward adaptation of the procedure developed previously to isolate self-cleaving RNAs (Pan & Uhlenbeck, 1992a), the selection described here has been much more efficient. Enrichment of P RNA substrates was more than 10 orders of magnitude in eight selection cycles, compared to 2−3 orders of magnitude for selecting Pb^{2+} cleavage motifs. Perhaps the most important reason for this high efficiency is the nature of the cleavage products. P RNA products contain 5′-phosphate and 3′-hydroxyl groups that can be directly religated without the interference of the background hydrolysis products, which contain 5′-hydroxyl and 2′,3′-cyclic phosphate groups. Although the background hydrolysis remains at ~1% to 5% of total circular RNA in each round, they will not be carried over to the next cycle of selection. On the other hand, this level of background strongly impaired the enrichment of Pb^{2+} cleavage motifs, whose products also contain 2′,3′-cyclic phosphate and 5′-hydroxyl groups. Like the selection experiments described previously (Pan & Uhlenbeck, 1992a), factors other than specific cleavage can play important roles in determining the outcome of the selection. Mostly notably, the efficiency to recircularize the cleavage product of the class I substrates constitutes a strong selection pressure.

Since the structure and/or sequence requirements of the selected substrates seem to differ significantly from the known RNase P substrates, there is an intriguing possibility that RNase P *in vivo* may process RNAs that have not yet been identified. Three natural substrates have been found so far for the *E. coli* P RNA complexed with its specific protein, C5. They include tRNA precursors and two other

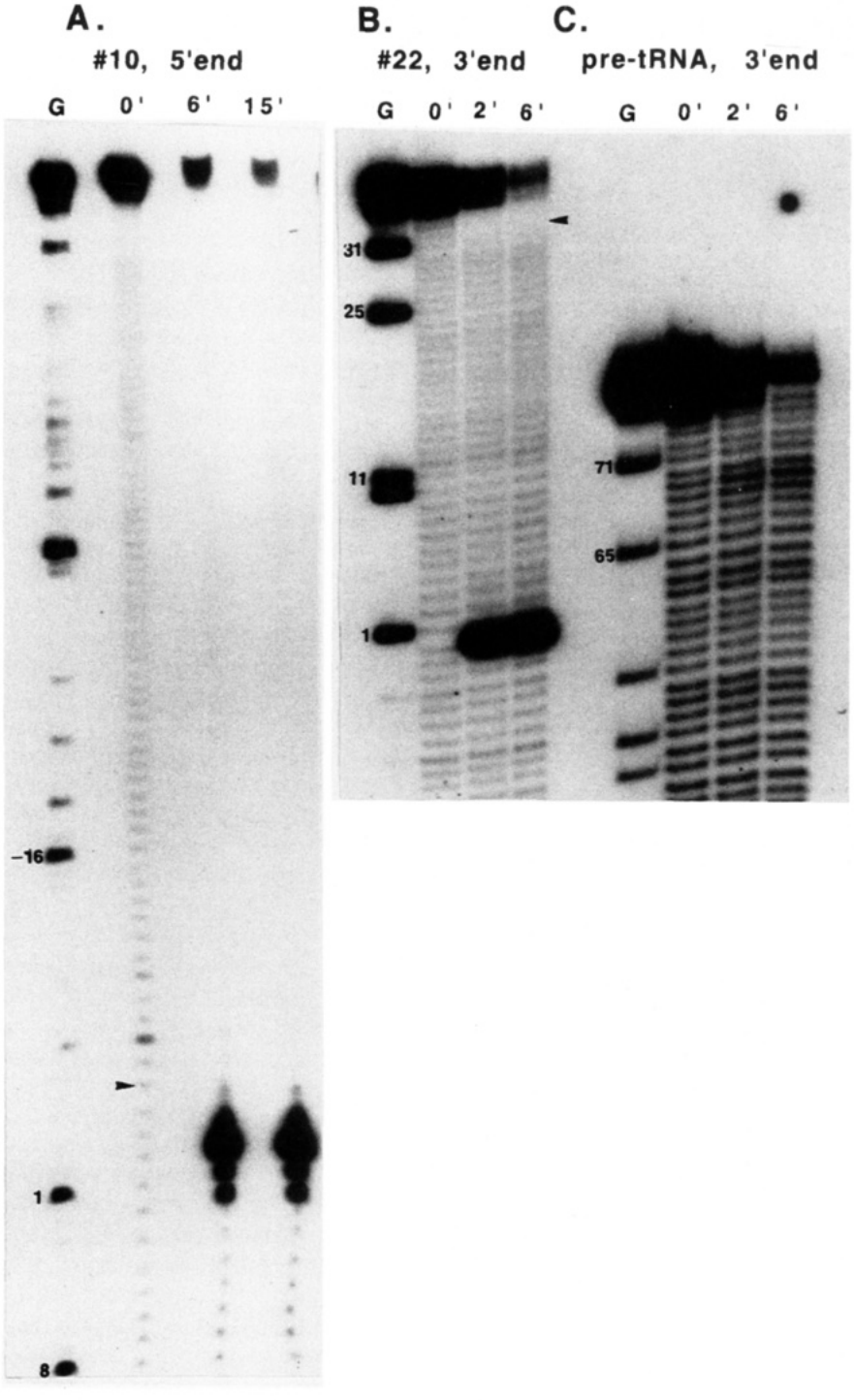

FIGURE 4: Terminal truncation experiments indicating the minimal sequence requirements of some selected variants. G: partial nuclease T1 digest of the labeled RNA. Time courses are in minutes. (A) 10, 5′ end; (B) 22, 3′ end; (C) pre-tRNA^{Phe}, 3′ end. The nucleotides 3′ to the cleavage site are numbered with positive signs. The end limits are indicated by arrowheads.

stable RNAs, 4.5S RNA (Peck-Miller & Altman, 1991; Altman et al., 1993) and 10Sa RNA (Komine et al., 1994). The RNA−protein complex, or the holoenzyme, has broadened substrate specificity compared to the P RNA alone, which is much less efficient at cleaving the 4.5S RNA precursor. Results from the *in vitro* selection experiment by Liu and Altman (1994) indicate that the *E. coli* RNase P holoenzyme can cleave, with improved efficiency, substrates that were selected by cleavage with P RNA alone. By assuming that this observation is also relevant for the *B. subtilis* holoenzyme, it is likely that the *B. subtilis* holoenzyme can also cleave the selected substrates efficiently. One approach to find these potential RNase P substrates based on the selected variants is to search for embedded RNA motifs in Genbank sequences, once the complete sequence and structural requirements for the *in vitro* selected substrates are known (Pan et al., 1991).

Finally, the finding that P RNAs can cleave substrates that are relatively unstructured invokes alternate ways to design guide RNAs that can be used to cleave undesirable RNAs *in vivo*. Ribozyme targeting involves using one externally introduced nucleic acid molecule to specifically recognize and cleave a designated mRNA. Currently, all ribozyme systems recognize their cellular RNA target through Wat-

son−Crick base pairs (Cech, 1992; Uhlenbeck, 1993). The P RNA substrates selected here are, however, sufficiently different to warrant further characterizations that may indicate means of recognizing target RNAs other than exclusively through Watson−Crick base pairing.

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REFERENCES

Altman, S., Kirsebom, L., & Talbot, S. (1993) *FASEB J. 7*, 7−14.
Bartel, D. P., & Szostak, J. W. (1993) *Science 261*, 1411−1418.
Beebe, J. A., & Fierke, C. A. (1994) *Biochemistry 33*, 10294−10304.
Cech, T. R. (1990) *Annu. Rev. Biochem. 59*, 543−568.
Cech, T. R. (1992) *Curr. Opin. Struct. Biol. 2*, 605−609.
England, T. E., Bruce, A. G., & Uhlenbeck, O. C. (1980) *Methods Enzymol. 65*, 65−75.
Forster, A., & Symons, R. H. (1987) *Cell 50*, 9−16.
Haas, E. S., Morse, D. P., Brown, J. W., Schmidt, F. J., & Pace, N. R. (1991) *Science 254*, 853−856.
Harris, M. E., Nolan, J. M., Malhotra, A., Brown, J. W., Harvery, S. C., & Pace, N. R. (1994) *EMBO J. 13*, 3953−3963.
Kirsebom, L. A., & Svard, S. G. (1994) *EMBO J. 13*, 4870−4876.
Komine, Y., Kitabatake, M., Yokogawa, T., Nishikawa, K., & Inokuchi, H. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9223−9227 (1994).
LaGrandeur, T. E., Huttenhofer, A., Noller, H. F., & Pace, N. R. (1994) *EMBO J. 13*, 3945−3952.
Liu, F., & Altman, S. (1994) *Cell 77*, 1093−1100.
McClain, W. H., Guerrier-Takada, C., & Altman, S. (1987) *Science 238*, 527−530.
Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15*, 8783−8798.
Nolan, J. M., Burke, D. H., & Pace, N. R. (1993) *Science 261*, 762−765.
Oh, B.-K., & Pace, N. R. (1994) *Nucleic Acids Res. 22*, 4087−4094.
Pace, N. R., & Smith, D. (1990) *J. Biol. Chem. 265*, 3587−3590.
Pan, T. (1995) *Biochemistry 34*, 902−909.
Pan, T., & Uhlenbeck, O. C. (1992a) *Biochemistry 31*, 3887−3895.
Pan, T., & Uhlenbeck, O. C. (1992b) *Nature 358*, 560−563.
Pan, T., & Zhong, K. (1994) *Biochemistry 33*, 14207−14212.
Pan, T., Gutell, R. R., & Uhlenbeck, O. C. (1991) *Science 254*, 1361−1364.
Peck-Miller, K. A., & Altman, S. (1991) *J. Mol. Biol. 221*, 1−5.
Perreault, J.-P., & Altman, S. (1992) *J. Mol. Biol. 226*, 399−409.
Reich, C., Olsen, G. J., Pace, B., & Pace, N. R. (1988) *Science 239*, 178−181.
Schlegl, J., Furste, J. P., Bald, R., Erdmann, V. A., & Hartmann, R. K. (1992) *Nucleic Acids Res. 20*, 5963−5970.
Smith, D., & Pace, N. R. (1993) *Biochemistry 32*, 5273−5281.
Strobel, S. A., & Cech, T. R. (1993) *Biochemistry 32*, 13593−13604.
Uhlenbeck, O. C. (1993) in *Antisense Research and Applications*, pp 83−96, CRC Press, Inc., Boca Raton, FL.
Uhlenbeck, O. C., & Gumport, R. I. (1982) in *The Enzymes*, Vol. 15, pp 31−58, Academic Press, New York.
Westhof, E., & Altman, S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5133−5137.

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