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Contribution of structural elements to *Thermus thermophilus* ribonuclease P RNA function

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We have performed a deletion and mutational analysis of the catalytic ribonuclease (RNase) P RNA subunit from the extreme thermophilic eubacterium *Thermus thermophilus* HB8. Catalytic activity was reduced 600-fold when the terminal helix, connecting the 5′ and 3′ ends of the molecule, was destroyed by deleting 15 nucleotides from the 3′ end. In comparison, the removal of a large portion (94 nucleotides, about one quarter of the RNA) of the upper loop region impaired function only to a relatively moderate extent (400-fold reduction in activity). The terminal helix appears to be crucial for the proper folding of RNase P RNA, possibly by orientating the adjacent universally conserved pseudoknot structure. The region containing the lower half of the pseudoknot structure was shown to be a key element for enzyme function, as was the region of nucleotides 328–335. Deleting a conserved hairpin (nucleotides 304–327) adjacent to this region and replacing the hairpin by a tetranucleotide sequence or a single cytidine reduced catalytic activity only 6-fold, whereas a simultaneous mutation of the five highly conserved nucleotides in the region of nucleotides 328–335 reduced catalytic activity by $>10^5$-fold. The two strictly conserved adenines 244 and 245 (nucleotides 248/249 in *Escherichia coli* RNase P RNA) were not as essential for enzyme function as suggested by previous data. However, additional disruption of two helical segments (nucleotides 235–242) adjacent to nucleotides 244 and 245 reduced activity by $>10^4$-fold, supporting the notion that nucleotides in this region are also part of the active core structure. Finally, we suggest that the severe interference with RNase P RNA function caused by many deletions supports the notion that the great bulk of its almost 400 nucleotides co-organize a single domain of higher order RNA structure.

Key words: deletion analysis/processing/RNase P/*Thermus thermophilus* HB8/tRNA

Introduction

Ribonuclease (RNase) P is a ubiquitous ribonucleoprotein particle which cleaves tRNA precursors to generate their mature 5′ termini. In eubacteria it is composed of a large RNA subunit ~400 nucleotides in size and a small basic protein of ~120 amino acids (Brown and Pace, 1992). RNA subunits of eubacterial RNase P enzymes were shown to be catalytically active in the absence of protein components (Guerrier-Takada *et al.*, 1983). Catalysis by RNA subunits from nuclear, mitochondrial or archaebacterial RNase P enzymes in the absence of associated proteins has not been demonstrated so far.

Recent studies on *Escherichia coli* RNase P RNA showed that catalytic activity can be largely restored by reconstitution of up to four subfragments (Guerrier-Takada and Altman, 1992). Some deletions of *E.coli* RNase P RNA affected activity in a substrate-dependent manner, i.e. abolished processing of a pre-tRNA^{Tyr}, but still allowed cleavage of the precursor of 4.5S RNA, and vice versa. Based on this study, approximately one third of the nucleotides of *E.coli* RNase P RNA were categorized as having less importance for enzyme function *in vitro* (Guerrier-Takada and Altman, 1992). Mutants of *E.coli* RNase P RNA lacking evolutionarily variable structural elements still retained high catalytic activities; some deletions, however, destabilized the RNA structure (Darr *et al.*, 1992). A simplified RNase P RNA (*Min*1, 263 nucleotides), consisting only of evolutionarily conserved features and lacking ~100 nucleotides, was shown to be highly active *in vitro* (Waugh *et al.*, 1989). However, the K_m of *Min*1 RNA for the pre-tRNA substrate was increased ~100-fold. Recently, a phylogenetically defined core structure of 192 nucleotides has been published for eubacterial RNase P RNA (Haas *et al.*, 1994). In a previous study of the RNase P RNA from *Bacillus subtilis* (Pace *et al.*, 1987; Waugh and Pace, 1993), most deletions decreased catalytic activity and many were found to reduce activity by $>10^4$-fold compared with the wild-type RNA in the *in vitro* test system used (Waugh and Pace, 1993). The authors concluded that no single nucleotide of *B.subtilis* RNase P RNA was absolutely required for catalysis.

RNase P RNAs from thermophilic eubacteria such as *Thermus thermophilus* (Hartmann and Erdmann, 1991), *Thermus aquaticus* and *Thermotoga maritima* (Brown *et al.*, 1993) were shown to be more thermostable than their counterparts from mesophilic eubacteria. In addition, RNase P RNA from *T.thermophilus* was less severely affected by Pb^{2+} ion-induced fragmentation (Ciesiolka *et al.*, 1994) than *E.coli* RNase P RNA. This suggested that structural alterations might be less detrimental to ribozyme function in the context of more stable overall structures of thermophilic RNase P RNAs. In addition, the potential to form alternative structures might be minimized in thermostable RNAs (Brown *et al.*, 1993), or activation energy barriers for the refolding of misfolded RNAs might be more easily overcome in the case of thermostable RNAs at elevated assay temperatures. Thus, a deletion analysis of *T.thermophilus* RNase P RNA would appear to be a particularly useful complement to the data obtained with *E.coli* (Guerrier-Takada and Altman, 1986,

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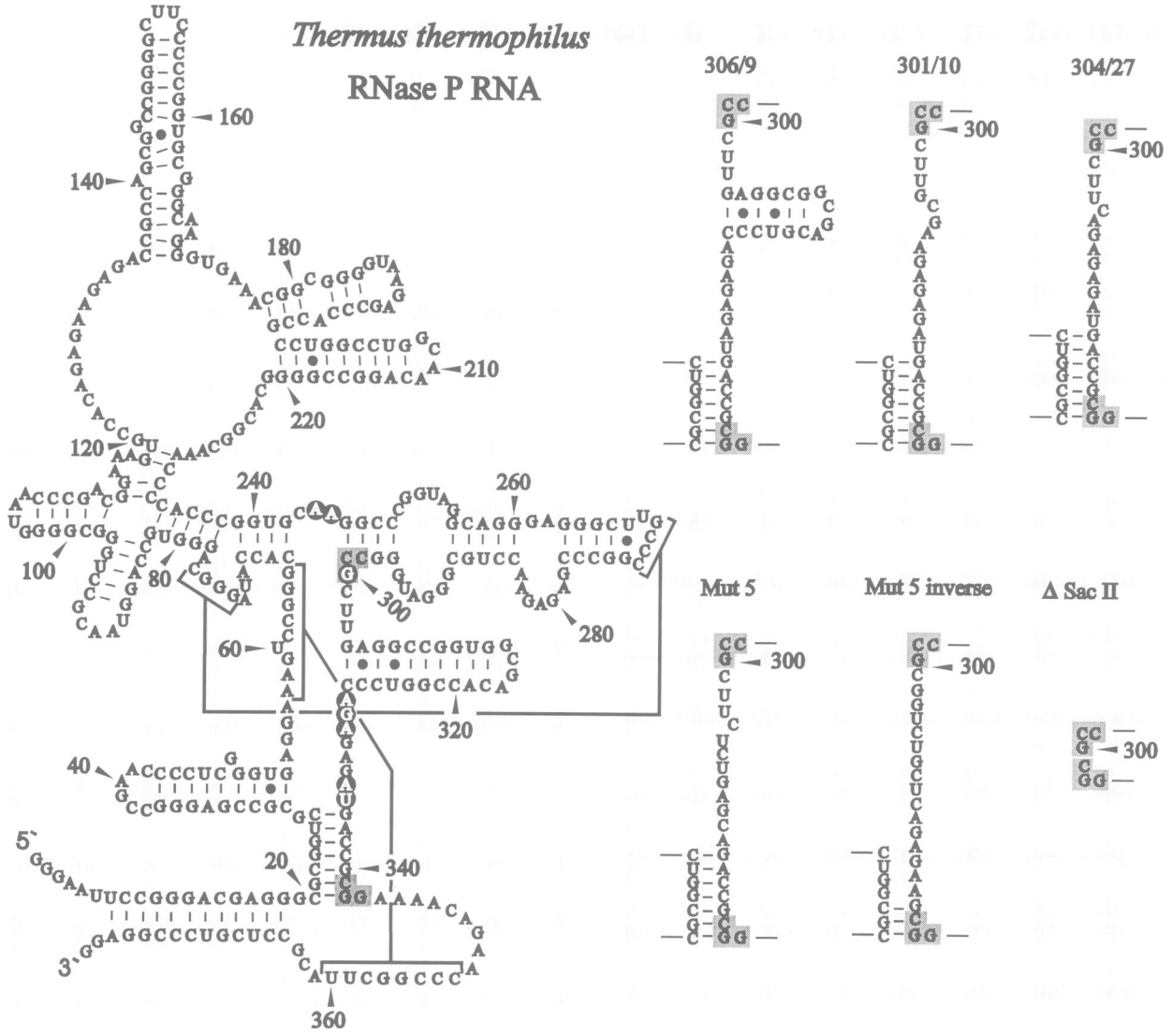

Fig. 1. Secondary structure of *T.thermophilus* RNase P RNA (Hartmann and Erdmann, 1991) according to the model of Haas *et al.* (1994). Nucleotides at positions 328, 329, 334 and 335 are invariant in eukaryotic and bacterial RNase P RNA subunits (Tranguch and Engelke, 1993); highlighted positions indicate nucleotides in the region between nucleotides 244 and 335 that are strictly conserved among eubacterial RNase P RNAs (Haas *et al.*, 1994). The right-hand side of the figure shows the sequences of *T.thermophilus* RNase P RNA variants in the region between nucleotides 300 and 341. Nucleotides which create the *Sac*II site in variant Δ*Sac*II are shaded in grey.

1992; Waugh *et al.*, 1989; Darr *et al.*, 1992; Talbot and Altman, 1994) and *B.subtilis* (Waugh and Pace, 1993) RNase P RNAs so as to delineate the structural elements that contribute to the catalytic function or maintenance of the catalytic core structure of this class of RNA enzymes.

Based on the data presented here, the following conclusions can be drawn. (i) A 600-fold reduction in the activity of RNase P RNA missing the 3′ terminal 15 nucleotides suggests that the terminal helix of RNase P RNA is involved in anchoring the adjacent universally conserved pseudoknot in the structure. (ii) The region containing the lower half of this pseudoknot structure is essential for enzyme function. (iii) The two consecutive adenines (nucleotides 244 and 245, Figure 1) in the vicinity of the 5′ end of bound tRNA, as inferred from cross-linking experiments (Burgin and Pace, 1990), may not be as essential for enzyme function as might have been suggested by the cross-linking data and by their strict conservation among eubacterial RNase P RNAs (Haas *et al.*, 1994); additional disruption of two helical segments (nucleotides 235–242) adjacent to nucleotides 244 and 245 resulted in a $>10^4$-fold reduced activity, suggesting the important role of structural elements in this region for catalytic function. (iv) Removal of a large proportion (94 nucleotides, about one quarter of the RNA) of the upper loop region impaired function to a relatively moderate extent (400-fold reduction in activity). Thus, the 'lower part' of RNase P RNA (comprising nucleotides ~1–120 and ~230–377) may be considered as the main catalytic domain, and the 'upper loop region' (nucleotides ~120–230) as an accessory second domain. (v) The region containing nucleotides 328–335, which includes four bases strictly conserved in prokaryotic and eukaryotic RNase P RNA subunits (Tranguch and Engelke, 1993), appears to be one key element for enzyme function; its exact spacing with respect to the structural elements of nucleotides 246–299 is less critical, since the hairpin comprising nucleotides 304–327 could be replaced by a tetranucleotide sequence or a single cytidine without reducing activity >6-fold. (vi) Evidence is lacking that inherent thermostability of

the *T.thermophilus* RNase P RNA alleviates detrimental effects on enzyme function caused by the structural alterations investigated here.

Results and discussion

To identify structural elements of RNase P RNA required for catalytic activity, altered *T.thermophilus* RNase P RNAs carrying deletions at the 5′ or 3′ ends were assayed with a pre-tRNA^{Gly} from the same organism (Schlegl *et al.*, 1992) as the substrate. At the beginning of this study, RNA fragments were exclusively synthesized from PCR templates by T7 RNA polymerase. Some of these RNAs with extensive terminal truncations showed residual processing activity in initial experiments. To apply a rigid test for activity, these RNA fragments were loaded onto denaturing polyacrylamide gels, lanes were sliced up after electrophoresis and gel pieces representing the different sections of the lane were eluted separately. RNA fractions from individual gel slices were then analysed for processing of pre-tRNA^{Gly}. To our surprise, fractions of main activity sometimes migrated above the fraction containing the RNA fragment of interest, as visualized by UV shadowing, or activities could not be recovered after this gel fractionation procedure. Hence, these activities were not considered to be authentic, since the possibility of trace contaminations with other RNase P RNA fragments could not be ruled out rigorously. Contaminations with other DNA fragments are a potential drawback of PCR-related methods, and this problem is likely to be even more severe with G/C-rich DNAs derived from *T.thermophilus*, as suggested by our observation that amplifications of *T.thermophilus* RNase P RNA templates give generally lower yields of specific DNA products than obtained in the corresponding PCR employing the *E.coli* RNase P RNA gene (unpublished results). Thus, we synthesized RNA fragments critical for the evaluation of low residual activities from cloned templates.

Among RNase P RNA variants with 3′ end deletions, only fragment nucleotides c1–362 (the prefix c indicates that the RNA was derived from a cloned template; Table I) gave authentic and reproducible processing activities, whereas fragments nucleotides c1–342 and c1–335 were unable to generate detectable amounts of mature tRNA^{Gly} (Figure 2; Table II) even after prolonged incubation overnight (note that a catalytic rate, k_{obs}, of 10^{-4} min^{-1} means 10% substrate converted to mature tRNA after 16 h at 55°C; 2–3% product formation was required for unambiguous detection). RNA nucleotides c1–362, in which the terminal helix is disrupted, showed a 600-fold reduction in activity (Table II), suggesting that the helix formed between the 5′ and 3′ ends functions as a molecular clamp of the RNase P RNA structure. RNA nucleotides c1–342 and c1–335 additionally lacked the lower part of the pseudoknot found at a similar location in all RNase P RNA subunits analysed so far (Forster and Altman, 1990; Tranguch and Engelke, 1993; Haas *et al.*, 1994). In contrast to our results, Waugh and Pace (1993) have reported that a deletion variant of *B.subtilis* RNase P RNA (lacking nucleotides 1–14 and 330–401) retains significant activity (up to 20% of that of the wild-type RNA), although missing the terminal helix and the lower part of the conserved pseudoknot. One should note that the region of nucleotides 348–360 (Figure 1) includes nine nucleotides strictly conserved among eubacteria, four of which are involved in the pseudoknot structure (Haas *et al.*, 1994).

Table I. 5′ and 3′ extensions of *T.thermophilus* RNase P RNA fragments

RNase P RNA fragments	Additional 5′ nucleotides	Additional 3′ nucleotides
c1–362	no	GGGGATC
c1–342	no	GGGGATC
c1–335	no	GGGGATC
1–327	no	no
1–299	no	no
c1–245	no	GGGTACCG
1–245	no	no
1–242	no	no
c1–242	no	GGGTACCG
c1–234	no	GGGGATC
c1–130	no	GGGGATC
c54–377	no	GGGGATC
c102–377	pGGGAATTCC	no
c171–377	no	ATC
c225–377	no	GGGGATC
c246–377	no	ATC
c300–377	pG	GGGGATC
328–377	pGG	no
336–377	pG	no

c, RNAs synthesized from cloned templates; without prefix, RNAs transcribed from PCR templates.

To characterize the role of internal sequence elements of *T.thermophilus* RNase P RNA, we reconstituted RNase P RNAs with internal sequence gaps by hybridizing pairs of *per se* inactive 3′ and 5′ fragments (Figure 2; Table II). This method has also been used in previous studies of *E.coli* (Guerrier-Takada and Altman, 1992) and *B.subtilis* RNase P RNA (Waugh and Pace, 1993). In this study, a preincubation step was performed to allow proper annealing of RNA fragments, and the optimal temperature for this step coincided with the assay temperature of 55°C. As observed in the study of *E.coli* RNase P RNA (Guerrier-Takada and Altman, 1992), combinations of fragments with large sequence redundancies, such as nucleotides c54–377 plus c1–335, c1–342 or c1–362 (Table II), yielded highly active complexes. This suggests that the steric constraints caused by sequence redundancies are of minor importance, and sequence overlaps apparently support the formation of highly active RNase P RNA complexes.

The complex of nucleotides c1–242/c300–377 lacking the authentic nucleotides 243–299 still retained residual processing activity (an ~2×10^3-fold reduction; Figure 2; Table II). The structural equivalent of nucleotides 246–299 (Figure 1) in *E.coli* RNase P RNA was shown recently to be disruptable without abolishing activity *in vitro* (Guerrier-Takada and Altman, 1992). In another study, the deletion of nucleotides 260–290 of *E.coli* RNase P RNA (roughly equivalent to nucleotides 256–289 in Figure 1), which are involved in a pseudoknot structure (Haas *et al.*, 1991), was shown to reduce substrate affinity by 100-fold and to confer temperature sensitivity on the RNA (Darr *et al.*, 1992). In addition, specific photoaffinity cross-links between tRNA 3′ ends and several RNase P RNAs were located in the region between nucleotides 246 and 299 (B.-K.Oh and N.R.Pace, personal communication). Based

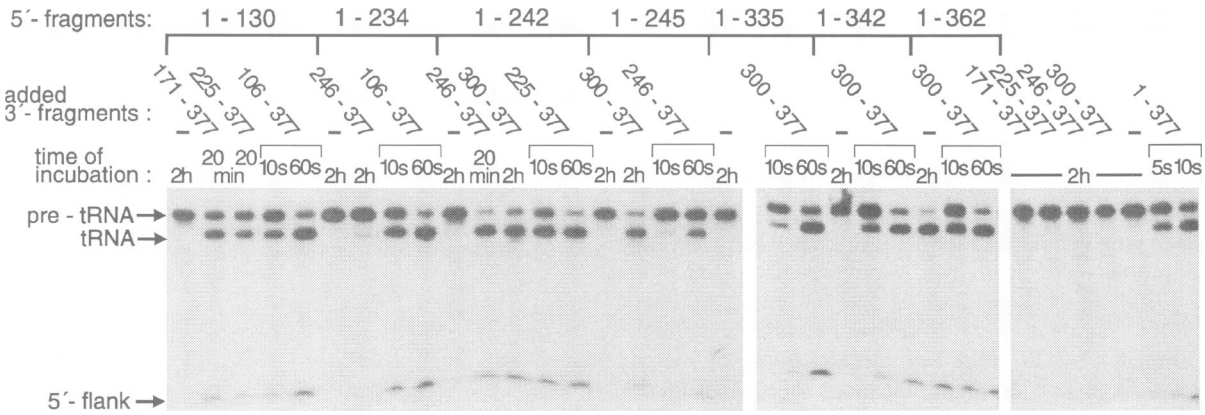

Fig. 2. Processing of pre-tRNA^{Gly} by cloned fragments of *T.thermophilus* RNase P RNA and combinations thereof (RNA fragments with the prefix c in Table I). The pre-tRNA^{Gly} substrate (Schlegl *et al.*, 1992) and RNase P RNA fragments were preincubated separately for 1 h at 55°C, and reactions were started by combining prewarmed enzyme and substrate mixtures (for details see Materials and methods). Cleavage assays contained 2.5 μM fragments or reconstituted RNase P RNA and 1–10 nM ^{32}P-labelled pre-tRNA^{Gly}. Incubation times (between 10 s and 2 h) are indicated above each lane. RNAs were separated on 10% polyacrylamide−8 M urea gels and visualized by autoradiography. The pre-tRNA^{Gly} substrate and products of the cleavage reaction, i.e. the mature tRNA and the 5′ flank, are marked by arrows.

Table II. Activity of *T.thermophilus* RNase P RNA fragments and combinations thereof

	Alone	1–130	1–234	1–242	1–245	1–299[a]	1–327[a]	1–335	1–342	1–362
Alone										0.017
1–377	11.0									
54–377		2.33						6.0	5.0	8.0
102–377		2.30	5.60							
171–377		0.043								
225–377		0.027		8.60						
246–377			0.001	0.13	0.4 (4)[b]					
300–377				0.006	0.006	0.16		1.6	1.6	3.3
328–377[a]							1.3	3.5		
336–377[a]								0.19		

Activities are given as substrate conversion per minute (k_{obs}; see Materials and methods). Experimental errors found in individual experiments were in the range ±5–50%.

[a]RNA fragments transcribed from PCR templates; all other activity values are based on experiments with RNA fragments synthesized from cloned templates.

[b]The value of 4 in parentheses is the k_{obs} obtained with RNA fragment 1–245 transcribed from a PCR template that lacks the 3′ extension present in the RNA fragment transcribed from the cloned template (Table I).

on these observations, sequences in the region of nucleotides 243–299, which are required for appreciable activity, appear to have a general role for tRNA interaction and/or structural stabilization in eubacterial RNase P RNAs. The internal loop region of *E.coli* RNase P RNA, corresponding to nucleotides 250–255/290–295 in *T.thermophilus* RNase P RNA (Figure 1), is also thought to accommodate a catalytically important Mg^{2+} binding site (Kirsebom and Svärd, 1993).

Cross-links, obtained with a tRNA carrying a photoreactive group at the 5′ terminus, have provided evidence that the two conserved adenines corresponding to positions 244 and 245 in Figure 1, which are strictly conserved among eubacterial RNase P RNAs (Haas *et al.*, 1994), are in the vicinity of the tRNA 5′ end in the tRNA−RNase P RNA enzyme−product complex (Burgin and Pace, 1990). Cross-links were also obtained in this region of *E.coli* RNase P RNA (corresponding to nucleotides 243–245 in Figure 1) with a tRNA carrying a photoreactive group at nucleotide 64 in the T stem (Nolan *et al.*, 1993). Thus, nucleotides 244 and 245 (Figure 1) might be part of the active site, although their exact location with respect to the pre-tRNA cleavage site has yet to be determined.

When we analysed reconstituted complexes of nucleotides c1–242/c246–377 (Table II; k_{obs} = 0.13 min^{-1}), where the two conserved adenines were replaced by eight nucleotides of unrelated sequence beyond nucleotide 242, and a second complex of nucleotides c1–245/c246–377 (k_{obs} = 0.4 min^{-1}), activities were significant (<100-fold reduced) and very similar for both fragment combinations. Reconstitution of nucleotides 1–245/c246–377, where the fragment nucleotides 1–245 lacked the 3′ extension of unrelated sequence added by transcription from the cloned DNA template (Table I), was more active (Table II; k_{obs} = 4.0 min^{-1}). Complex nucleotides 1–242/c246–377, missing nucleotides 243–245 completely, had the same activity (~0.13 min^{-1}) as the combination of nucleotides c1–242/c246–377 (data not shown). In conclusion, alterations in the region of nucleotides 243–245 have significant effects on activity, although the presence of nucleotides 243, A244 and A245 is not as crucial for enzyme function *in vitro* as one might expect for nucleotides involved in active site geometry. Consistently, an A → G mutation at the position in *E.coli* RNase P RNA corresponding to A245 in *T.thermophilus* RNase P RNA only moderately affected *in vitro* activity (Lumelsky and Altman, 1988).

Two central helical segments of RNase P RNA were disrupted by combining fragments nucleotides c1–234 and c246–377. This reduced catalytic activity ~10^4-fold (Figure 2; Table II), supporting the importance of structural elements in this region. Similar though more extensive deletions in the corresponding region of *B.subtilis* RNase P RNA led to the most dramatic reductions in activity in the study by Waugh and Pace (1993).

The extensive deletion of nucleotides 131–224, which includes three bases strictly conserved among eubacteria (Haas *et al.*, 1994), reduced activity 'only' 400-fold (Table II). Hence, this region, comprising about one quarter of the RNA, seems to be of lower importance for RNase P RNA function *in vitro* in the absence of the protein subunit. One should note that 100- to 1000-fold reductions in activity have been classified as moderate effects in the deletion study presented here, whereas differences in kinetic constants by factors of 5–10 are often employed to characterize the RNase P RNA cleavage reaction. Guerrier-Takada and Altman (1992) still observed processing of pre-4.5S RNA, but not of a pre-tRNA^{Tyr}, in the presence of C5 protein with an *E.coli* RNase P RNA variant missing nucleotides 94–204 (corresponding to nucleotides 85–199 in Figure 1). Since this deletion overlaps with the deletion of nucleotides 131–224 of *T.thermophilus* RNase P RNA, this provides further evidence that structural elements of the 'upper loop region' of eubacterial RNase P RNAs contribute relatively little to the productive enzyme–substrate interaction.

The complex of nucleotides 1–299/c300–377, although lacking any deletions or sequence redundancies except for one additional G at the 5′ end of nucleotides 300–377 (see Materials and methods), had the lowest activity (70-fold reduced compared with RNase P RNA; Table II) among reconstituted RNase P RNAs without sequence gaps. Thus, activity can be profoundly affected even by minor structural perturbations in this region. This effect may be related to the base identity of G300, which is invariable among eubacterial RNase P RNAs (Haas *et al.*, 1994). A similar situation was found for the combination nucleotides c1–335/336–377 (60-fold reduced; Table II), where the sugar phosphate backbone was disrupted at the strictly conserved U335. In addition, sequence gaps in the region of nucleotides 300–335, as represented by the combinations of nucleotides 1–299/328–377 or 1–327/336–377, abolished measurable catalytic activity. These results indicate that the catalytic RNA is particularly sensitive to structural alterations in the region of nucleotides 300–335. Several nucleotides strictly conserved among eubacterial RNase P RNAs (Haas *et al.*, 1994) reside in this region, i.e. A328, G329, A330, A334 and U335 (Figure 1), all of which, except for A330, are invariant in prokaryotic as well as eukaryotic RNase P RNA subunits (Tranguch and Engelke, 1993). Several cross-links between a tRNA carrying an azidophenacyl photoreactive group at the 5′ terminus, and RNase P RNAs from *E.coli*, *Chromatium vinosum* and *B.subtilis*, were localized (Burgin and Pace, 1990) in the region corresponding to nucleotides 330–333 (Figure 1). Reconstituted *B.subtilis* RNase P RNAs, missing the nucleotides corresponding to A328, G329, A330 and A334 in Figure 1, were reported to have extremely low or no activity (Waugh and Pace, 1993). However, one circularly permuted, unimolecular RNA carrying a sequence gap of 37 nucleotides in this region was found to retain residual activity (10^3-fold reduced; Waugh and Pace, 1993). The authors reasoned that the absence of sequence elements in this region may prevent folding of RNA fragments into an active complex.

Table III. Kinetic constants for processing of pre-tRNA^{Gly} by altered *T.thermophilus* RNase P RNAs

RNase P RNA	K_m (μM)	k_{cat} (min^{-1})
wt	0.8 (±0.2)	20 (±5)
306/9	10 (±3)	3.5 (±0.5)
301/10	7 (±0.5)	5 (±2)
304/27	7 (±2)	10 (±2)
Mut5	nd	1×10^{-3} ($\pm3\times10^{-4}$)[a]
Mut5 inv	nd	–
Δ*Sac*II	nd	–

nd, not determined; –, no product detectable after 16 h.
[a]Estimated on the basis of single turnover kinetics (see Materials and methods).

One structural element in the corresponding region of *T.thermophilus* RNase P RNA is the hairpin comprising nucleotides 304–327 (Figure 1). The hairpin is highly conserved in RNase P RNA subunits, though it is shorter in RNAs from Gram-positive bacilli (Brown and Pace, 1992). During this study, two natural eubacterial RNase P RNAs from *Chlorobium limicola* and *Chlorobium tepidum* were reported to have the hairpin comprising nucleotides 304–327 (Figure 1) substituted by a single C residue (Haas *et al.*, 1994). The kinetic behaviour of these RNase P RNAs was very similar to that of *E.coli* RNase P RNA in the multiple turnover reaction under conditions of 25 mM Mg^{2+}/1 M NH_4^+ and using a pre-tRNA^{Asp} as the substrate. In contrast, replacing the hairpin by a single C in *E.coli* RNase P RNA led to a 60-fold increase in K_m under the same *in vitro* assay conditions. The K_m of the altered *E.coli* RNase P RNA could be restored to that of the wild-type RNA by increasing the NH_4Cl concentrations to 3 M, suggesting that electrostatic repulsion of phosphate groups can be counteracted by high ionic strength. As yet undefined structural features of RNase P RNAs from *Chlorobium* species appear to compensate for the absence of the hairpin structure in these RNAs (Haas *et al.*, 1994).

Since we could not exclude that pairs of RNA fragments with deletions in the region of nucleotides 300–335 failed to associate, we analysed this part of the RNA in detail using monomolecular *T.thermophilus* RNase P RNA. For this purpose, the corresponding DNA sequence was deleted by PCR techniques (see Materials and methods), thereby creating a *Sac*II restriction site (Figure 1). Sequence variants in this region were obtained by cloning different oligonucleotide cassettes into the *Sac*II site (Figure 1). Altered *T.thermophilus* RNase P RNAs, which carried a hairpin of reduced size (variant 306/9) or in which the hairpin was replaced by a tetranucleotide sequence (variant 301/10) or a single C residue (variant 304/27), behaved similarly to the wild-type RNA in the multiple turnover cleavage reaction of pre-tRNA^{Gly} under our standard conditions of 0.1 M Mg^{2+}/0.1 M NH_4^+ (Table III). Catalytic rates were reduced at most 6-fold and K_m values of the multiple turnover reaction were increased up to a

factor of 10–15 compared with the wild-type RNA under standard assay conditions (Table III). An increase in k_{cat}, as observed for the *E.coli* variant (Haas *et al.*, 1994) corresponding to *T.thermophilus* variant 304/27, was not observed with the thermostable RNA. This is attributable to the fact that product release is not rate limiting in the processing reaction catalysed by wild-type *T.thermophilus* RNase P RNA under the standard assay conditions applied, as inferred from identical catalytic rates in the single and multiple turnover reaction (data not shown). The observation that the hairpin could be replaced by a single or four nucleotides without dramatically affecting activity points to a considerable adaptability of the RNA chain at this location in order to fulfil the so far unknown spatial constraints in this region of RNase P RNA. The role of the hairpin nucleotides 304–327 may be to properly fix adjacent single-stranded regions in the RNA structure.

We also changed nucleotides 328, 329, 330, 334 and 335 in variant 304/27, thus affecting all nucleotides that are strictly conserved in this region of eubacterial RNase P RNAs. The resulting RNA Mut5, as well as the variant Mut5 inverse (which was obtained by cloning the oligonucleotide cassette in the reverse orientation into the *Sac*II site), were tested in the single turnover processing reaction of pre-tRNA^{Gly} (Table III). The catalytic activity of RNA Mut5 was extremely low, but could be stimulated 5-fold by increasing the concentration of NH_4OAc to 1.5 M (data not shown). For RNase P RNA variants Mut5 inverse and ΔSacII lacking nucleotides 301–340, authentic processing activity could not be determined unequivocally and, unlike the wild-type *T.thermophilus* RNase P RNA and variant Mut5, neither RNA was stimulated by higher NH_4OAc concentrations.

Parallel *in vitro* and *in vivo* experiments with point mutants of *E.coli* RNase P RNA also support the notion that the intricate local arrangement in the region of nucleotides 328–335 is crucial for enzyme function. In addition, structural probing did not reveal any profound effects of point mutations in this region on more distal parts of the RNA structure (unpublished results). Point mutations G → A329 (Shiraishi and Shimura, 1988) and A → U334 (Baer *et al.*, 1988) of *E.coli* RNase P RNA were reported to severely impair activity. For the A329 mutant, a 400-fold reduction in the rate of pre-tRNA cleavage was reported (Shiraishi and Shimura, 1988). The higher-order structure of *E.coli* RNase P RNA was found to be unaffected by the G → A329 mutation in chemical modification studies (Shiraishi and Shimura, 1988). Interestingly, nucleotides corresponding to positions 332 or 333 in Figure 1 were the only positions in different RNase P RNAs whose protection from chemical modification was specific for pre-tRNA; a precursor segment of 2–4 nucleotides sufficed to confer this protection (T.E.LaGrandeur and N.R.Pace, personal communication). In summary, we conclude that the region comprising nucleotides 328–335 is crucial to maintaining enzyme function. Of course, a direct involvement of this part of eubacterial RNase P RNA in active site chemistry cannot be inferred from the data discussed here.

RNase P RNAs from thermophilic bacteria have been shown to be inherently more resistant to thermal denaturation (Hartmann and Erdmann, 1991; Brown *et al.*, 1993) and inactivation by Pb^{2+}-induced fragmentation (Ciesiolka *et al.*, 1994). This suggests that the *T.thermophilus* RNase P RNA might be able to overcome structural destabilizations caused by mutations better than mesophilic RNase P RNAs. However, the majority of alterations in *T.thermophilus* RNase P RNA investigated here caused severe reductions in catalytic activity similar to those observed with mesophilic RNase P RNAs (Guerrier-Takada and Altman, 1992; Waugh and Pace, 1993). This provides further evidence that eubacterial RNase P RNAs are optimized 'RNA folding units' with a complex higher-order structure essentially defined by the phylogenetically conserved core structure (Haas *et al.*, 1994). Our results and those of a previous study on *E.coli* RNase P RNA (Guerrier-Takada and Altman, 1992) may justify the consideration of the 'lower part' of RNase P RNA (comprising nucleotides ~1–120 and ~230–377) as the main catalytic domain, and the 'upper loop region' (nucleotides ~120–230) as an auxiliary second domain. Structural elements, such as the conserved nucleotides A244/A245 (Figure 1), appear to provide only a partial contribution to enzyme function. Compared with corresponding deletions in RNase P RNA from the mesophile *B.subtilis* (Waugh and Pace, 1993), alterations such as the removal of the region enclosing the lower part of the conserved pseudoknot were even more detrimental to enzyme function of the thermophilic RNA. In summary, substantial evidence is lacking that the inherent thermostability of the *T.thermophilus* RNase P RNA may generally counteract losses of function caused by structural alterations. The reason may be that multiple intra- and intermolecular interactions, dispersed about the entire RNase P RNA core structure, act in a highly cooperative manner to yield the productive enzyme–substrate complex. Even small structural alterations may perturb the intricate arrangement of the phosphate backbone within the polyanionic RNA molecule, leading to electrostatic repulsion of phosphates and thus to inactive conformations. It seems likely that the key towards understanding this RNA enzyme lies in the three-dimensional organization of its sugar phosphate backbone.

Materials and methods

Preparation of RNA

RNAs were synthesized as run-off transcripts with T7 RNA polymerase in the presence of 9 mM GMP such that >90% of transcripts carry a single phosphate at their 5′ ends, essentially as described recently (Schlegl *et al.*, 1992; Hardt *et al.*, 1993; Ciesiolka *et al.*, 1994). T7 template DNAs for altered RNase P RNAs were constructed by PCR techniques (see below), and were subsequently cloned into the vector pUC18 and verified by DNA sequencing. RNAs were transcribed by run-off transcription with T7 RNA polymerase after linearization of plasmids with *Bam*HI or *Sst*I, yielding the RNA 3′ extensions listed in Table I. The template DNA for RNA c102–377 was obtained by eliminating an *Eco*RI–*Bst*EII fragment from plasmid pT7M1HB8 (Hartmann and Erdmann, 1991) and religation of the truncated plasmid after a fill-in reaction using Klenow polymerase. DNA amplification assays were performed with DNA polymerase from *Pyrococcus furiosus* as recommended by the manufacturer (Stratagene). 5′ primers for *T.thermophilus* RNase P DNA fragments (in RNA sense) carried 17 nucleotides of T7 promoter sequence (5′-TAATACGACTCACTATA-3′) and the following nucleotides of RNase P DNA (according to the numbering system shown in Figure 1): 54–84, 131–154, 171–201, 225–255, 246–276, 300–329, 328–359 and 336–365; the last three encoded one or two (328–359) additional Gs at the 5′ end of the T7 transcript (Table I). The 5′ primer 1 for fragments encoding the intact 5′ terminus of RNase P RNA was 5′-*TTTAATACGACTCACTATA*GG**GAATTC**C-3′ (T7 promoter sequence in italic, *Eco*RI site in bold), which is identical

to the 5′ region of the RNase P RNA gene under the control of the T7 promotor in plasmid pT7M1HB8. 3′ primers were complementary to the following nucleotides of *T.thermophilus* RNase P DNA: 130–107, 234–208, 242–216; 245–219, 299–264, 327–298, 335–306, 362–330 and 377–343. Approximately 1–10 ng of plasmid were used as template DNA for amplification. *T.thermophilus* RNase P RNA lacking nucleotides 301–340 (variant Δ*Sac*II, Figure 1) was constructed by a two-step PCR using plasmid pT7M1HB8 carrying the wild-type gene as the template; in the first PCR, a 5′ portion of the RNase P RNA gene was amplified with primer 1 (see above) and primer 2 (5′-GGGTTCTGTTTTCCG-*CGGCCCATCCCGCAGGTTCTCTCGGGCCGGGCA*; italic sequence complementary to nucleotides 300–268 in Figure 1); in the second PCR, the RNA sense strand of the first PCR was annealed with primer 3 (5′-CGC**GGATCC**TCCGGGACGAGGCGTAAGCC*GGGTTCTGTTTTC*-*CGCGGCCC*; *Bam*HI site in bold) by means of the 21 complementary nucleotides in italic in primer 3, and recessed 3′ ends were filled in with DNA polymerase from *P.furiosus*; then primer 1 was added to amplify the full-length product. The PCR product was digested with *Eco*RI and *Bam*HI, cloned into the vector pT7T318U (Pharmacia) and verified by DNA sequencing. The recombinant plasmid (pΔ*Sac*II) was employed to insert different oligonucleotide cassettes into its unique *Sac*II site, yielding altered RNase P RNAs shown in Figure 1.

Assays of catalytic activity

T.thermophilus pre-tRNA^{Gly} internally labelled with ^{32}P was used as substrate (Schlegl *et al.*, 1992). Cleavage assays with (altered) RNase P RNAs, as well as with RNase P RNA fragments and combinations thereof, were performed in 1× TAMN [50 mM TrisOAc, 100 mM $Mg(OAc)_2$, 100 mM NH_4OAc and 2 mM EDTA, pH 7.0 at 25°C; ~pH 6.3 at 55°C] at 55°C. Enzyme RNAs and the pre-tRNA^{Gly} substrate were preincubated separately for 60 min under respective reaction conditions. Reactions were started by combining prewarmed enzyme and substrate mixtures. Cleavage reactions were stopped by adding 1 vol of loading buffer (67% formamide, 0.3× TBE, 2.7 M urea, 100 mM EDTA) and shock-freezing in liquid nitrogen. Single turnover (pre-steady state) experiments ($E >> {}^{*}S$) were performed with trace amounts (1–10 nM) of ^{32}P-labelled pre-tRNA^{Gly} and excess amounts of RNase P RNA variants Mut5, Mut5 inverse and Δ*Sac*II essentially as described (Been *et al.*, 1992; Smith and Pace, 1993). Pseudo first-order rate constants for cleavage (k_{obs}, min^{-1}) were calculated at each enzyme concentration by the slope of a least-fit squares linear regression of a plot of $-\ln[S_t - S_{(t\to\infty)}]/[1\text{-}S_{(t\to\infty)}]$ versus time; S_t is the fraction of substrate at time t and $S_{(t\to\infty)}$ corresponds to the fraction of substrate at the end point (~0.05, since ~95% of the pre-tRNA^{Gly} could be converted to mature tRNA after 1 h incubation with wild-type *T.thermophilus* RNase P RNA). Values of k_{obs} were plotted as k_{obs} versus $[E]$. Hyperbolic curves were analysed by visual inspection, yielding an approximation of k_{react}, the rate constant for the single turnover reaction at saturating $[E]$. The rate constant k_{react} is expected to equal k_{cat} (the Michaelis–Menten rate constant for the multiple turnover reaction at $S >> E$) since product release is not rate limiting in the reaction catalysed by *T.thermophilus* RNase P RNA at 0.1 M Mg^{2+} and 0.1 M NH_4^+ (data not shown). Since RNase P RNA variants Mut5, Mut5 inverse and Δ*Sac*II could not be analysed by multiple turnover kinetics due to their low activities and expected high K_m values, k_{cat} values in Table III were estimated on the basis of single turnover experiments (performed in 1× TAMN buffer). For cleavage assays with the truncated RNase P RNAs and fragment combinations summarized in Table II, concentrations of enzymatic RNA were adjusted to 2.5 μM in the presence of 1–10 nM pre-tRNA^{Gly}; under these conditions, the single turnover cleavage rate (k_{obs}) for the *T.thermophilus* wild-type RNase P RNA is ~11 min^{-1}.

Multiple turnover (steady state) experiments with wild-type RNase P RNA and variants 306/9, 301/10 and 304/27 (Table III) were performed with a ≥40-fold excess of pre-tRNA over RNase P RNA. The substrate concentration was varied in six or seven increments to cover a range from 0.1 to 5×K_m. Values of K_m and k_{cat} ($v_{max}/[E]_{total}$) for multiple turnover reactions were determined by Lineweaver–Burk plots. Values for k_{cat} in multiple turnover reactions correspond to moles of substrate converted per mole of enzyme per minute.

Kinetic constants in Tables II and III were derived from several independent experiments. Deviations found in individual experiments indicate that errors range between ±5 and 50%.

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