

# Phylogenetic comparative mutational analysis of the base-pairing between RNase P RNA and its substrate

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## ABSTRACT

We have studied the base-pairing between the 3'-terminal CCA motif of a tRNA precursor and RNase P RNA by a phylogenetic mutational comparative approach. Thus, various derivatives of the *Escherichia coli* tRNA<sup>Ser</sup>Su1 precursor harboring all possible substitutions at either the first or the second C of the 3'-terminal CCA motif were generated. Cleavage site selection on these precursors was studied using mutant variants of M1 RNA, the catalytic subunit of *E. coli* RNase P, carrying changes at positions 292 or 293, which are involved in the interaction with the 3'-terminal CCA motif. From our data we conclude that these two C's in the substrate interact with the well-conserved G292 and G293 through canonical Watson-Crick base-pairing. Cleavage performed using reconstituted holoenzyme complexes suggests that this interaction also occurs in the presence of the C5 protein. Furthermore, we studied the interaction using various derivatives of RNase P RNAs from *Mycoplasma hyopneumoniae* and *Mycobacterium tuberculosis*. Our results suggest that the base-pairing between the 3'-terminal CCA motif and RNase P is present also in other bacterial RNase P-substrate complexes and is not limited to a particular bacterial species.

**Keywords:** ribozyme; RNase P; tRNA precursors; tRNA processing

## INTRODUCTION

RNase P is an endoribonuclease responsible for the maturation of the 5'-termini of tRNA molecules. In bacteria this enzyme consists of an RNA subunit and a protein subunit; in *Escherichia coli* these are designated M1 RNA and C5, respectively. The catalytic subunit of RNase P is the RNA, and under appropriate in vitro conditions it cleaves a tRNA precursor at the correct position in the absence of the protein (Guerrier-Takada et al., 1983). The C5 protein is, however, essential for activity in vivo (for recent reviews, see Altman et al., 1995; Kirsebom, 1995; Pace & Brown, 1995). Compensatory mutation analysis has been shown to be a powerful method for investigation of interactions within as well as between RNA molecules (see, e.g., Gesteland & Atkins, 1993). Recently we used this approach and demonstrated the importance of Watson-Crick complementarity between the two well-conserved residues G292 and G293 in M1 RNA, and the two C's (+74C

and +75C) in the 3'-terminal CCA sequence of a tRNA precursor (Kirsebom & Svärd, 1994). We used mutant M1 RNAs carrying a G to C change at either position 292 or 293 in M1 RNA and precursors harboring a +74G or a +75G. Here we have extended this analysis by changing the residues at these positions in the enzyme and in the substrate to any of the other nucleotides. Furthermore, we have investigated the general existence of this interaction by studying the cleavage pattern for wild-type and mutant RNase P RNA derivatives derived from *Mycoplasma hyopneumoniae* and *Mycobacterium tuberculosis* (Svärd et al., 1994, and unpubl. data). Because the C5 protein is essential for activity in vivo, we also studied whether this interaction is present in reconstituted holoenzyme-tRNA precursor complexes. From our data, we conclude that the well-conserved G292 and G293 residues in M1 RNA interact with the two C's in the 3'-terminal CCA sequence in a tRNA precursor through canonical Watson-Crick base-pairing and that this interaction is present in the holoenzyme-substrate complex. Our results also suggest that the residues that correspond to G292 to G293 in other bacterial RNase P RNAs interact with the 3'-terminal CCA motif of the precursor in a way similar as G292 and G293 in M1 RNA.

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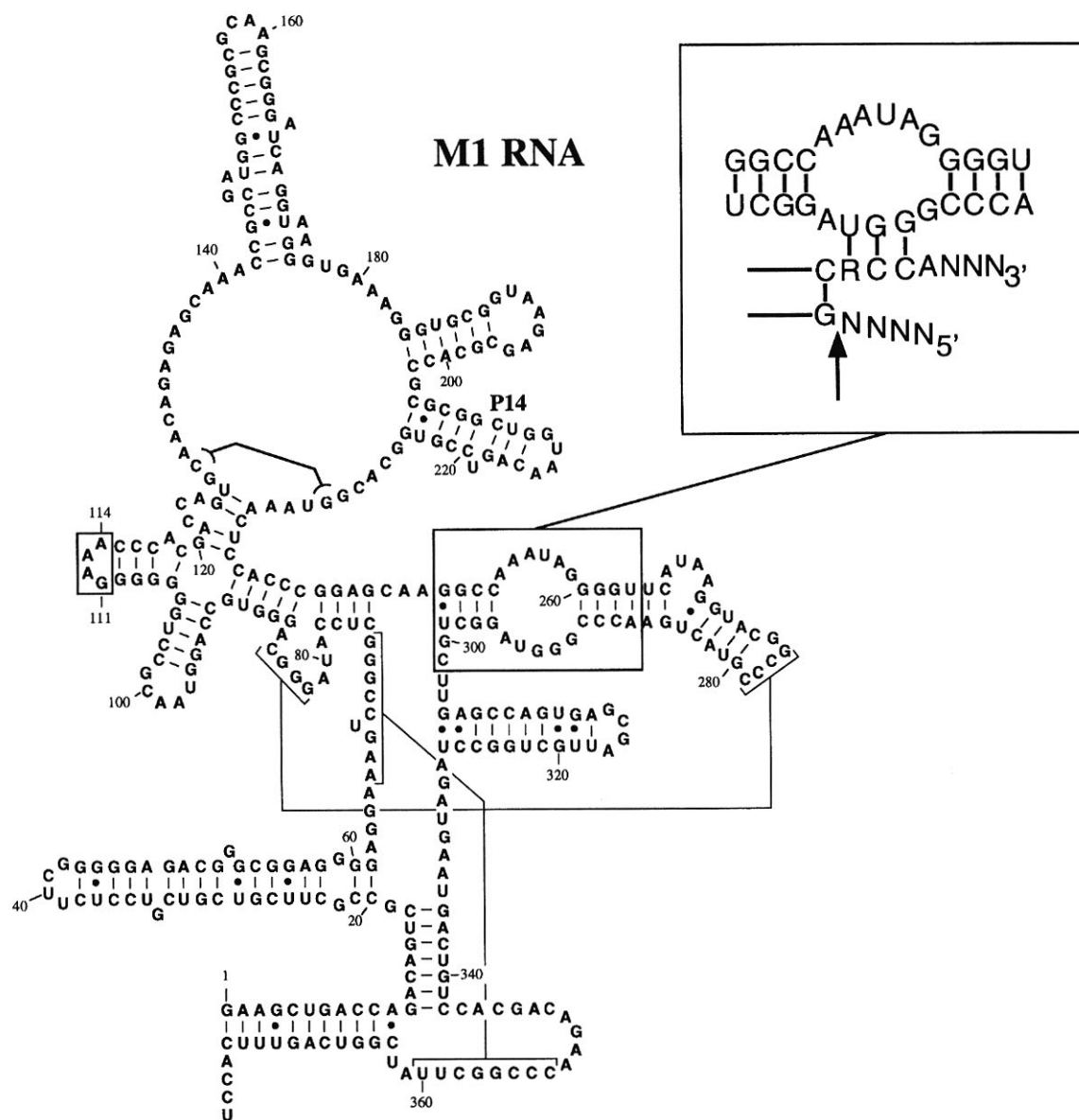
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## RESULTS

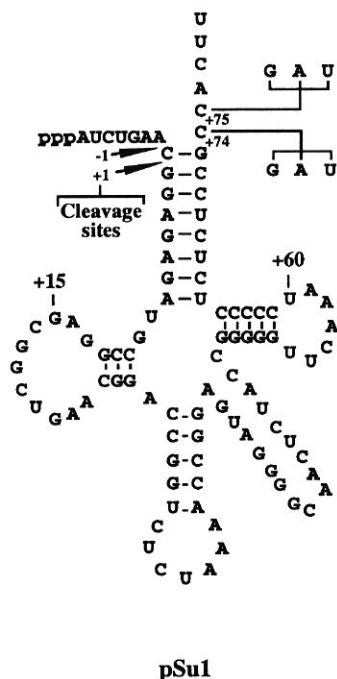
### The 3'-terminal CCA motif interacts with M1 RNA through canonical Watson-Crick base-pairing

Cleavage of the tRNA<sup>Ser</sup>Su1 precursor (pSu1) at the correct position is dependent on an intact 3'-terminal CCA motif (Kirsebom & Svärd, 1993, 1994). The predictions from our suggestion that G292 and G293 in M1 RNA interact through Watson-Crick base-pairing with the two C's in the CCA motif at the 3'-termini of a

tRNA precursor (Fig. 1) are that any substitution in the precursor resulting in disruption of this interaction would give miscleavage, and an introduction of the corresponding compensatory mutation in M1 RNA would suppress this miscleavage such that cleavage occurs preferentially at the correct position. To test this, we generated M1 RNA derivatives and various mutant pSu1 precursors that carried all possible variations: at positions 292 and 293 in the ribozyme and at positions +74 and +75 in the tRNA precursor. These changes are illustrated in Figures 2 and 3, and our results are



**FIGURE 1.** A secondary structure of M1 RNA, the catalytic subunit of *E. coli* RNase P, according to Brown et al. (1994) and Mattsson et al. (1994). The inset shows a model of the interaction between the 3'-terminal "RCCA motif" of a precursor and M1 RNA in the enzyme-substrate complex. As discussed in the text, the boxed residues G111-A114 have been positioned close to the T-loop in the two existing 3D models of M1 RNA in complex with its substrate (Harris et al., 1994; Westhof & Altman, 1994).

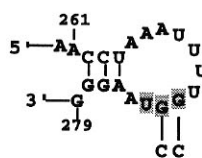
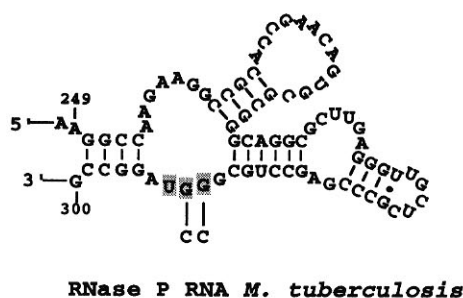
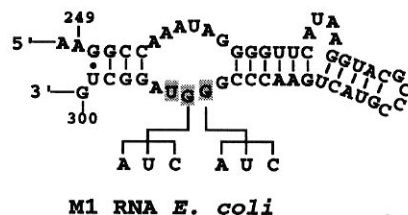


**FIGURE 2.** Illustration of the secondary structure of the *E. coli* tRNA<sup>Ser</sup>Su1 precursor (pSu1) used in this study. The mutant derivatives of pSu1 were changed at positions +74 or +75 as indicated. Arrows show the RNase P cleavage sites at -1 and at +1. Numbering is according to Steinberg et al. (1993).

summarized in Table 1. The position of cleavage (at -1 or at +1) was based on the mobility of the 5'-cleavage fragment; a typical analysis is shown in Figure 4.

As expected, a substitution of either the +74C or the +75C resulted in significant miscleavage at -1 by wild-type M1 RNA, whereas cleavage at the correct position was restored by using mutant M1 RNAs carrying the corresponding compensatory change. Note that restoration of cleavage site selection required a specific change in M1 RNA; for example, a substitution of G292 does not result in cleavage at the correct position on precursors carrying changes at +74. This demonstrates that this interaction between M1 RNA and its substrate is specific. From these data we conclude that G292 and G293 in M1 RNA interact with +74C and +75C in the substrate through canonical Watson-Crick base-pairing. In those combinations where we had a +75U/A and A/U292, we noted that the suppression of the miscleavage was lower in comparison to other combinations. This might be due to the fact that the +75/292 interaction is the closing base pair of this short helix (see Fig. 1), and a weak base pair at this position might result in breathing and therefore increased miscleavage.

It has been demonstrated that miscleavage of a tRNA precursor can be suppressed at high ionic strength (Guerrier-Takada et al., 1989). Thus, we analyzed the cleavage pattern at an increased  $\text{NH}_4^+$  concentration using some of our mutant precursors and M1 RNA de-

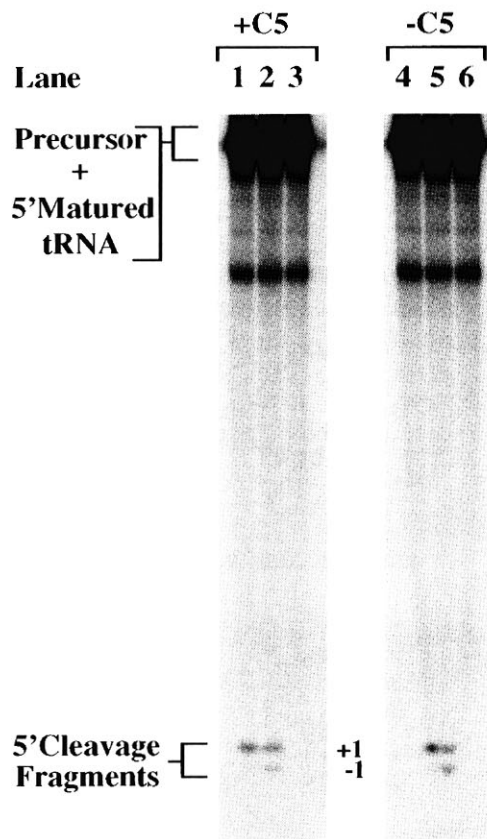


**FIGURE 3.** Structural comparison of the region in the different RNase P RNAs that are involved in base-pairing with the 3'-terminal CCA motif of a precursor tRNA. The various substitutions in the different RNase P RNAs were introduced at the positions indicated in the figure. For details, see the text.

rivatives carrying changes at relevant positions. As shown in Table 2, we observed a similar cleavage pattern with respect to the preference of cleavage site also at higher ionic strength. Thus, the base-pairing between M1 RNA and its substrate is not sensitive to the ionic strength, at least not at the concentration we have used in this report. This is in keeping with the findings of Kufel and Kirsebom (1994), who used model tRNA precursors that were cleaved at two positions and demonstrated that an increase in the concentration of  $\text{NH}_4^+$  did not influence cleavage site selection.

#### Interaction between the 3'-terminal CCA motif of pSu1 and RNase P RNA from other species

To investigate whether this base-pairing is also present in other RNase P RNA-substrate complexes, we decided to analyze cleavage site selection on the *E. coli* derived pSu1 precursor using various derivatives of *M. tuberculosis* and *M. hyopneumoniae* RNase P RNAs. Both these species are distantly related to *E. coli*, and these RNase P RNA molecules show structural differ-



**FIGURE 4.** Cleavage of the wild-type *E. coli* pSu1 precursor by different wild-type RNase P RNA derivatives in the absence and in the presence of the *E. coli* C5 protein. The experiments were performed at 37 °C as described in the Materials and methods section. The final concentrations of reactants were as follows: precursor, 0.09 pmol/ $\mu$ L; wild-type *E. coli* RNase P RNA (M1 RNA), 0.082 pmol/ $\mu$ L in the absence of C5 and 0.0016 pmol/ $\mu$ L in the presence of C5; wild-type *M. hyopneumoniae* RNase P RNA (HyoRNase P RNA), 0.36 pmol/ $\mu$ L in the absence of C5 and 0.0073 pmol/ $\mu$ L in the presence of C5. The cleavage products were separated from the precursors on an 18% denaturing polyacrylamide gel. Lanes 1–3, cleavage in the presence of C5. Lane 1, M1 RNA, duration of cleavage (dc) = 20 min; lane 2, HyoRNase P RNA, dc = 68 min; lane 3, no RNase P RNA added, only C5, dc = 68 min. Lanes 4–6, cleavage in the absence of C5 in Buffer B. Lane 4, M1 RNA, dc = 15 min; lane 2, HyoRNase P RNA, dc = 15 min; lane 3, no RNase P RNA added, dc = 15 min. The presence of the RNA fragment observed in all lanes is due to the way the precursor was prepared.

ences near the region which in M1 RNA base-pairs with the 3'-terminal CCA motif of the substrate. Characterization of wild-type RNase P RNA derived from *M. hyopneumoniae* (HyoRNase P RNA) has been published previously (Svärd et al., 1994), whereas characterization of *M. tuberculosis* RNase P RNA (MycRNase P RNA) will be published later (Mattsson et al., in prep.). The secondary structures of the region of interest in the different RNase P RNAs, including the changes we introduced, are depicted in Figure 3. The results of this analysis are shown in Table 2. The cleavage patterns using these RNase P RNA derivatives were studied under high ionic strength (Buffer B; see Materials and

methods) because the efficiency of cleavage by these RNase P RNA derivatives is improved under these conditions (Svärd et al., 1994; data not shown for MycRNase P RNA).

Both of these wild-type RNase P RNAs cleaved the mutant pSu1 precursors carrying a substitution at either +74 or +75 significantly at the –1 position. This suggests that the two C's in the 3'-terminal CCA motif of this tRNA precursor also influence the selection of the cleavage site by other bacterial RNase P RNAs. Surprisingly, wild-type HyoRNase P RNA cleaved the wild-type *E. coli* precursor significantly both at position +1 and at position –1. A similar result was obtained when cleavage was performed in the "low" ionic strength buffer, Buffer A (data not shown). The miscleavage of the precursor carrying +74G was suppressed as a result of substitution of a G to a C at the position corresponding to 293 in M1 RNA, i.e., at positions 313 and 273 in MycRNase P RNA and HyoRNase P RNA, respectively (see Fig. 3). Miscleavage of pSu1-G75 was suppressed by a substitution of G272 and G312 in these RNase P RNAs to C's, but also by the HyoRNase P RNA variant carrying C273. In fact, this latter mutant suppressed the miscleavage of pSu1-G75 more efficiently than the C272 variant. We also noted a slight suppression of the miscleavage of, in particular, pSu1-G74 by MycRNase P RNA harboring a C312, which was not observed using RNase P RNA derived from *E. coli*, as shown above. These data indicate a less specific interaction between these RNase P RNAs and the pSu1 precursor, particularly in the case of HyoRNase P RNA. This might be due to the fact that we used a wild-type tRNA precursor derived from *E. coli* and/or attributed to the structural differences in the region of interest in these RNase P RNA species (see also below). From these data it appears that at least MycRNase P RNA interacts with its substrate in a way similar to M1 RNA.

#### The base-pairing is also present in the holoenzyme–substrate complex

The experiments described so far were performed in the absence of the protein subunit of RNase P, C5. The C5 protein is essential for cleavage in vivo, therefore it was of interest to investigate whether the base-pairing is also present in the enzyme–substrate (ES) complex in the presence of C5. Hence, we used some of the mutant substrates and M1 RNAs and studied cleavage site selection in the presence of C5. The results suggested that an intact 3'-terminal CCA motif is also required for correct cleavage of the pSu1 precursor in cleavage by the wild-type *E. coli* reconstituted holoenzyme (Table 3). This is in keeping with our previous findings (Kirsebom & Svärd, 1993). Furthermore, suppression of cleavage at the –1 position was observed by the mutant M1 RNAs harboring a substi-

**TABLE 1.** Summary of the cleavage sites using different pSu1 precursors and various M1 RNA derivatives in the absence of the C5 protein.<sup>a</sup>

M1 RNA	Cleavage site	Precursor						
		wt	+74G	+74A	+74U	+75G	+75A	+75U
Wild type	-1		97	94 ± 6	64 ± 7	92	87	76 ± 12
	+1	<u>100</u>	3	6 ± 6	36 ± 7	8	13	24 ± 12
C293	-1	37	4	80		95	87	
	+1	63	<u>96</u>	20		5	13	
U293	-1	90		16 ± 8	82 ± 10		88 <sup>b</sup>	89 <sup>b</sup>
	+1	10		<u>84 ± 8</u>	18 ± 10		12 <sup>b</sup>	11 <sup>b</sup>
A293	-1	100		97	16 ± 13		91 <sup>b</sup>	93 <sup>b</sup>
	+1			3	<u>84 ± 13</u>		9 <sup>b</sup>	7 <sup>b</sup>
C292	-1	93 <sup>c</sup>	95 ± 6			14		
	+1	7 <sup>c</sup>	5 ± 6			<u>86</u>		
U292	-1	100		91	90	83	57	80 ± 6
	+1			9	10	17	<u>43</u>	20 ± 6
A292	-1	100		90	86	92	89	62
	+1			10	14	8	11	<u>38</u>

<sup>a</sup> The frequency of cleavage at the different positions is given as a percentage value, and each value is an average of several independent experiments. The experimental error was ≤5 percentage units except in those cases indicated in the table. The experiments were performed in Buffer A as described in the Materials and methods section. The underlined numbers represent cleavage at the correct position in those cases where a Watson-Crick base pair can be formed.

<sup>b</sup> Numbers based on one experiment.

<sup>c</sup> Numbers taken from Kirsebom and Svärd (1994).

**TABLE 2.** Summary of the cleavage sites using different pSu1 precursors and various RNase P RNA derivatives.<sup>a</sup>

Ribozyme	Cleavage site	Precursor		
		wt	+74G	+75G
M1 RNA				
	Wild type			
Wild type	-1	95	91	
	+1	<u>99</u>	5	9
C293	-1	38	8	91
	+1	62	<u>92</u>	9
C292	-1	97	91	10
	+1	3	9	<u>90</u>
<i>M. tuberculosis</i> RNase P RNA				
	Wild type <sup>c</sup>			
Wild type <sup>c</sup>	-1	84	34	
	+1	<u>100</u>	16	66
C313	-1	24	2	46
	+1	76	<u>98</u>	54
C312	-1	30	47	19
	+1	70	53	<u>81</u>
<i>M. hyopneumoniae</i> RNase P RNA				
	Wild type			
Wild type	-1	42	85	69
	+1	<u>58</u>	15	31
C273	-1	37	26	12
	+1	63	<u>74</u>	88
C272	-1	67	80	52
	+1	33	20	<u>48</u>

<sup>a</sup> The frequency of cleavage at the different positions is given as a percentage value, and each value is an average of several independent experiments. The experimental error was ≤4 percentage units. The experiments were performed in Buffer B as described in the Materials and methods section. The underlined numbers represent cleavage at the correct position in those cases where a Watson-Crick base pair can be formed.

tution of G293 or G292. Similar to what was observed in the absence of C5, specific changes were required to suppress miscleavage of pSu1-G74 and pSu1-G75, respectively. These data therefore suggest that the

**TABLE 3.** Summary of the cleavage sites using different pSu1 precursors and various M1 RNA and HyoRNase P RNA derivatives in the presence of the *E. coli* C5 protein.<sup>a</sup>

Ribozyme	Cleavage site	Precursor		
		wt	+74G	+75G
M1 RNA				
	Wild type			
Wild type	-1		75	73
	+1	<u>100</u>	25	27
C293	-1	27	11 ± 11	76
	+1	73	<u>89 ± 11</u>	24
C292	-1	55	67	9
	+1	45	33	<u>91</u>
<i>M. hyopneumoniae</i> RNase P RNA				
	Wild type			
Wild type	-1	25	92	91
	+1	<u>75</u>	8	9
C273	-1	32	30	67
	+1	68	<u>70</u>	33
C272	-1	18	90	53
	+1	82	10	<u>47</u>

<sup>a</sup> The frequency of cleavage at the different positions is given as a percentage value, and each value is an average of several independent experiments. The experimental error was ≤4 percentage units except in those cases indicated in the table. The underlined numbers represent cleavage at the correct position in those cases where a Watson-Crick base pair can be formed.



Watson–Crick interaction between residues G292 and G293 in M1 RNA and the two C's in the CCA motif is also present in the reconstituted holoenzyme ES complex.

The C5 protein derived from *E. coli* forms functional complexes with RNase P RNAs derived from other species (Altman et al., 1995, and references therein). Our previous studies showed that HyoRNase P RNA interacts with C5, generating an active enzyme (Svärd et al., 1994). The data of Vioque et al. (1988) suggest that the C5 protein influences the folding of *E. coli* RNase P RNA. Addition of C5 has also been demonstrated to suppress miscleavage of a truncated tRNA precursor by *E. coli* RNase P RNA (Guerrier-Takada et al., 1989). Thus, in an attempt to investigate whether the miscleavage of the wild-type pSu1 precursor by this RNase P RNA is related to its folding, we generated functional hybrid holoenzymes between the various HyoRNase P RNA derivatives and the *E. coli* C5 protein. As shown in Table 3 and Figure 4, the wild-type pSu1 precursor was still significantly miscleaved at the  $-1$  position. One reason for this might be that the *E. coli* C5 protein is unable to promote a correct folding of the HyoRNase P RNA (for alternative explanations, see below). Cleavage of the various precursor derivatives with different hybrid holoenzymes showed cleavage patterns similar to those that had been observed in the absence of C5 with the exception that a significant reduction in the suppression of cleavage at  $+1$  of pSu1-G75 by the C273 variant was observed in the presence of C5 (compare Tables 2 and 3). From the combined data, it appears that HyoRNase P RNA interacts with C74 in pSu1 in a way similar to M1 RNA, whereas the interaction with C75 is less clear.

## DISCUSSION

### RNase P interacts with its substrate through canonical Watson–Crick base-pairing

We previously showed that a deletion or substitution of the two C's in the terminal CCA sequence of the precursor to tRNA<sup>Ser</sup>Su1 resulted in significant miscleavage at the  $-1$  position. Based on the results presented in this report, where we performed a complete mutational analysis of the residues constituting this interaction, we conclude that M1 RNA base-pairs with the two C's in the CCA motif at the 3'-end of the tRNA<sup>Ser</sup>Su1 precursor through canonical Watson–Crick base-pairing. As previously suggested, this interaction is present irrespective of precursor given that the precursor carries a 3'-terminal CCA motif (Kirsebom & Svärd, 1994). Recent data show that residue U294 in M1 RNA interacts with tRNA precursors harboring a purine at the position ( $+73$ ) immediately preceding the 3'-terminal CCA sequence (below we will refer to this as the "RCCA motif"), thereby extending the inter-

action between M1 RNA and its substrate by one base pair (Tallsjö et al., 1996; see also Fig. 1). Our findings are in keeping with cross-linking and footprinting, as well as modification interference studies that suggest that the 3'-end of a precursor tRNA is in close contact with residues in this internal loop of M1 RNA as well as with the corresponding residues in other bacterial RNase P RNAs (Knap et al., 1990; LaGrandeur et al., 1994; Oh & Pace, 1994). The results from the two latter reports together with the data presented here, where we used various derivatives of RNase P RNAs derived from sources other than *E. coli*, suggest that this Watson–Crick interaction also exists in other bacterial RNase P RNA–tRNA precursor complexes. The GGU-motif in this region of RNase P RNA is not universal; however, most bacterial RNase P RNAs carry a GG-motif in this domain of the molecule (Brown et al., 1994). The structure of this region in cyanobacteria RNase P RNA is clearly different and does not contain this motif (Vioque, 1992), suggesting that these RNase P RNA variants interact with the substrate differently than does *E. coli* RNase P RNA. This might be related to the fact that most tRNA genes derived from cyanobacteria characterized so far do not encode the 3'-terminal CCA sequence (Steinberg et al., 1993).

Cleavage by various *E. coli* reconstituted holoenzymes resulted in cleavage patterns similar to those observed in the reactions with M1 RNA alone. Recently it was shown that the precursor of a bacteriophage antisense RNA is a substrate for RNase P. Efficient *in vitro* and *in vivo* processing of this precursor is dependent on an intact 3'-terminal CCA motif (Hartmann et al., 1995). The combined data therefore suggest that the Watson–Crick interaction between RNase P and its substrates plays an important role *in vivo*.

RNase P RNA is the only naturally isolated "trans-acting" ribozyme identified so far. However, "cis-acting" ribozymes can be modified to act in "trans"; one example is the *Tetrahymena* ribozyme, L-21 *Sca* I, which catalyzes site-specific cleavage of RNA oligonucleotides. Several similarities have emerged between the L-21 *Sca* I and the RNase P RNA cleavage reactions (Cech, 1993). The L-21 *Sca* I reaction starts with base-pairing between the oligonucleotide and an internal guide sequence (IGS), resulting in an RNA helix (P1). This helix is then docked into the active site of the ribozyme in a proposed induced-fit conformational change (Herschlag, 1992). Substrate misdocking into alternative registers results in miscleavage of the substrate (Strobel & Cech, 1994), and base substitutions in the P1-helix induced miscleavage (Murphy & Cech, 1989; Pyle et al., 1994). This is analogous to the effects we have described here when base-pairing between RNase P RNA and the 3'-terminal "RCCA motif" is disrupted. We have proposed earlier that one function of the base-pairing interaction is to anchor the substrate to the ribozyme (Kirsebom & Svärd, 1994). An induced-fit type of re-

action for M1 RNA has also been discussed (Guerrier-Takada et al., 1989; Kirsebom & Svård, 1993). This is in keeping with the suggestion that RNase P RNA undergoes conformational changes during the reaction cycle (Beebe & Fierke, 1994; Hardt et al., 1995). Thus, disrupted anchoring of the substrate to the ribozyme can result in misdocking of the cleavage site in the active site to an alternative register, producing miscleavage. Consistent with this is the finding that the structural arrangement at and near the cleavage site in the ES complex is dependent on the "RCCA-M1 RNA" interaction (Kufel & Kirsebom, 1996; Kufel & Kirsebom, unpubl.). Hence, our findings extend the observed similarities between L-21 Sca I and RNase P RNA.

Cleavage at the correct position occurs even though the 3'-terminal CCA of a tRNA precursor has been deleted or substituted (Green & Vold, 1988; Guerrier-Takada et al., 1989; Svård & Kirsebom, 1992). There are several determinants on a tRNA precursor important for the localization of the cleavage site (reviewed by Kirsebom, 1995). Disruption of the interaction with the "RCCA motif" resulted in miscleavage of a chimeric tRNA precursor when the amino acid acceptor-stem derived from the pSu1 precursor was transplanted into the precursor to tRNA<sup>Tyr</sup>Su3. The resulting precursor harbored an A at the -2 position. Changing this residue to a G resulted in cleavage preferentially at the correct position in spite of the absence of the "RCCA-M1 RNA" interaction (Kirsebom & Svård, 1993). Thus, other determinants can apparently compensate for the loss of this interaction. We also note the importance of the nucleotide at the -2 position in the processing of tRNA<sup>Met</sup> precursors in vivo (Meinzel & Blanquet, 1995).

### Cleavage and RNase P RNA structure

We observed that the wild-type *E. coli* tRNA<sup>Ser</sup>Su1 precursor was cleaved with approximately the same frequency at position +1 and at position -1 using HyoRNase P RNA. This was observed at low and at high ionic strength as well as in the presence of the *E. coli* C5 protein. HyoRNase P RNA alone cleaves the tRNA<sup>Tyr</sup>Su3 precursor and a precursor to tRNA<sup>His</sup> at the correct positions, whereas addition of the *E. coli* C5 protein resulted in some miscleavage of both of these precursors (Svård et al., 1994, and unpubl. results). Cleavage at the correct sites on these latter precursors does not depend on the "RCCA-M1 RNA" interaction, whereas correct cleavage of the tRNA<sup>Ser</sup>Su1 precursor does (Green & Vold, 1988; Kirsebom & Svård, 1993, 1994; this report). The structure in the region of HyoRNase P RNA that interacts with the 3'-terminal RCCA is clearly different compared with the same region in M1 RNA (Fig. 3). It has been suggested that the tertiary

folding of these regions in RNase P RNA derived from *E. coli* and *Bacillus subtilis* is similar (Haas et al., 1991) where the latter has a secondary structure similar to HyoRNase P RNA (Svård et al., 1994). Nevertheless, it is conceivable that the miscleavage of this precursor is due to the way the "RCCA motif" interacts with the *M. hyopneumoniae* RNase P RNA. This is to some extent supported by our finding that the HyoRNase P RNA carrying the C273 substitution in the RNA-alone reaction suppresses the miscleavage of both pSu1-G74 and pSu1-G75. We also have to consider that we used an *E. coli* tRNA precursor. Comparison of the gene encoding tRNA<sup>Ser</sup>Su1 with the gene encoding a tRNA<sup>Ser</sup> iso-acceptor in *Mycoplasma capricolum* and *Mycoplasma mycoides* (Samuelsson et al., 1985; Muto et al., 1990) revealed no differences at and near the cleavage site that could explain the observed cleavage pattern of the pSu1 precursor used here. However, there are differences at other positions, in particular at position +9 as well as in the D- and T-loops. Modification interference studies suggest that the nucleotide at +9 and residues in the T-loop are important for efficient RNase P cleavage (Kahle et al., 1990; Thurlow et al., 1991; Gaur & Krupp, 1993). In addition, a substitution of the G57 in the T-loop of the *E. coli* tRNA<sup>Tyr</sup>Su3 precursor results in miscleavage by *E. coli* RNase P (Svård & Kirsebom, 1993). Based on cross-linking data, the T-loop has been positioned close to nucleotides G111-A114 in M1 RNA in the two existing 3D models of M1 RNA in complex with its substrate (Harris et al., 1994; Westhof & Altman, 1994). These residues are part of a stem and loop structure (see Fig. 1), and in all mycoplasma RNase P RNAs characterized so far, the equivalent structure is represented by a "mini-helix" (Svård et al., 1994; Mattsson et al., in prep.). The presence of this "mini-helix" might therefore result in a different alignment of the *E. coli* pSu1 precursor, which consequently results in miscleavage by HyoRNase P RNA. Hence, it is conceivable that a tRNA<sup>Ser</sup> precursor of mycoplasma origin is cleaved only at the correct position. Furthermore, HyoRNase P RNA might not be folded in an optimal way even in the presence of the *E. coli* C5 protein, which could be a reason for our observations. This is supported to some extent by the finding that the precursors to tRNA<sup>Tyr</sup>Su3 and tRNA<sup>His</sup> were miscleaved by HyoRNase P RNA as a result of addition of the *E. coli* C5 protein (see above).

### The 3'-terminal CCA of a tRNA plays a crucial role in the interaction with other RNA as well as with proteins

The regulation of the expression of tyrosyl-tRNA synthetase in *B. subtilis* involves uncharged tRNA. Here the ACC at the 3'-end of tRNA<sup>Tyr</sup>, together with the anticodon, base-pairs with nucleotides in the leader se-

quence of the tyrosyl-tRNA synthetase messenger RNA (Grundy & Henkin, 1993; Grundy et al., 1994). A functionally important Watson-Crick base pair between the first C in the "RCCA motif" at the 3'-end of a tRNA and G2252 of 23S rRNA has recently been demonstrated (Samaha et al., 1995). Crystal structures of aminoacyl-tRNA-synthetase in complex with its cognate aminoacylated tRNA as well as the structure of the ternary EF-Tu, phe-tRNA<sup>Phe</sup>, and GTP-analogue complex show that the 3'-terminal CCA motif also plays a significant role in the interaction with proteins (Moras, 1994; Nissen et al., 1995). Taken together with the results presented in this report, these findings clearly demonstrate that interactions between tRNA and other RNA molecules as well as with proteins are partly mediated through interactions involving the well-conserved 3'-terminal CCA sequence that is common among all tRNA molecules.

RNase P recognizes and cleaves most if not all tRNA precursors in a cell. Several determinants on a tRNA precursor dictate the selection of the RNase P cleavage site: the 3'-terminal "RCCA motif" (see above); the nucleotide 3' of the cleavage site (at +1), which usually is a guanosine, as well as nucleotides at positions -2 and -1; and the length of the amino acid acceptor- and T-stems. In addition, cross-linking and modification interference experiments suggest that RNase P is in contact with the conserved -GUUCR- motif in the T-loop. In fact, substitution of either of the underlined residues in a tRNA precursor results in a decreased efficiency of cleavage by RNase P (for a review, see Kirsebom, 1995). Elongation factor Tu also recognizes and binds to all elongator tRNAs in a cell except tRNA<sup>Sec</sup> (Baron & Böck, 1995). Here, important close contact points are the 3'-terminal CCA, the guanosine at the 5'-end, and the -GU- motif of the conserved -GUUCR- sequence in the T-loop (Nissen et al., 1995). In addition, both these enzymes can use as substrate a stem and loop structure that corresponds to the acceptor-stem stacked on the T-stem (McClain et al., 1987; Nazarenko & Uhlenbeck, 1995). Thus, there are obvious similarities in the way RNase P and EF-Tu interact with their respective substrates. It will therefore be interesting to see whether there are also structural similarities between RNase P and elongation factor Tu.

## MATERIALS AND METHODS

### Plasmid construction and generation of tRNA precursors and mutant M1 RNAs

The construction of genes encoding some of the tRNA<sup>Ser</sup>Su1 derivatives (pSu1, pSu1-G74, and pSu1-G75) as well as the wild-type M1 RNA, M1<sub>C292</sub>RNA, and M1<sub>C293</sub>RNA behind the T7 promoter have been described elsewhere (Vioque et al., 1988; Kirsebom & Svärd, 1994). The wild-type RNase P RNA derived from *M. hyopneumoniae* has been described by

Svärd et al. (1994), whereas cloning and characterization of the gene encoding *M. tuberculosis* RNase RNA will be published elsewhere (Mattsson et al., in prep.).

The other base-substituted variants of pSu1 used in the present study were constructed using the polymerase chain reaction (PCR) (Saiki et al., 1988) according to Kirsebom and Svärd (1994). The various base substitutions in the different RNase P RNA genes were introduced using the Pharmacia Biotech USE Mutagenesis Kit according to the manufacturer's instructions, using plasmids carrying the corresponding wild-type RNase P RNA genes behind the T7 promoter as templates. The different gene constructs were verified by DNA sequencing (Sanger et al., 1977).

The precursor and the various RNase RNA genes carried appropriate restriction sites to generate the desired precursors and ribozymes after cleavage with the respective restriction enzyme and transcription with T7 DNA-dependent RNA polymerase (Milligan et al., 1987; Vioque et al., 1988).

### RNase P RNA assay

RNase P RNA activities were monitored as previously described in detail (Guerrier-Takada et al., 1983, 1988; Vioque et al., 1988; Kirsebom & Svärd, 1992). The reaction conditions were as follows. Buffer A (our standard assay buffer in the absence of the protein subunit of RNase P) was made up of 50 mM Tris-HCl (pH 7.5), 100 mM NH<sub>4</sub>Cl, 5% (w/v) polyethylene glycol 6000, and 100 mM MgCl<sub>2</sub>. Buffer B contained 50 mM Tris-HCl (pH 8.0), 800 mM NH<sub>4</sub>Cl, 0.05% Nonidet P-40, and 100 mM MgCl<sub>2</sub> (Reich et al., 1988). Buffer C (our standard assay buffer for the reconstituted holoenzyme) was 50 mM Tris-HCl (pH 7.5), 100 mM NH<sub>4</sub>Cl, and 10 mM MgCl<sub>2</sub>. In a typical assay, we mixed enzyme, which had been preincubated at 37 °C for 7 min, with substrate prewarmed for 2 min at the same temperature. The duration of incubation varied between 5 and 68 min, depending on the RNase P RNA derivative as well as the reaction conditions (see also legend of Fig. 4). The amount of enzyme in the RNase P RNA alone reaction varied from approximately equimolar amounts to approximately a 40-fold excess over substrate, depending on RNase P RNA and reaction conditions, whereas in the presence of C5, substrate was in excess over enzyme (approximately fivefold for the M1 RNA variants and approximately equimolar amounts in the cases where the HyoRNase P RNA derivatives were used).

Purified *E. coli* C5 protein was kindly provided by Dr. S. Altman and Ms. A. Tallsjö.

The positions of the cleavage sites were inferred from the mobility of the 5'-cleavage product, and calculation of the frequency of cleavage at the different positions was done as described elsewhere (Kirsebom & Svärd, 1993, 1994). The cleavage site is defined as the site where initial cleavage occurs.

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