The EMBO Journal vol.13 no.20 pp.4870–4876, 1994

Base pairing between *Escherichia coli* RNase P RNA and its substrate

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Communicated by I.Mattaj

Base pairing between the substrate and the ribozyme has previously been shown to be essential for catalytic activity of most ribozymes, but not for RNase P RNA. By using compensatory mutations we have demonstrated the importance of Watson–Crick complementarity between two well-conserved residues in *Escherichia coli* RNase P RNA (M1 RNA), G292 and G293, and two residues in the substrate, +74C and +75C (the first and second C residues in CCA). We suggest that these nucleotides base pair (G292/+75C and G293/+74C) in the ribozyme–substrate complex and as a consequence the amino acid acceptor stem of the precursor is partly unfolded. Thus, a function of M1 RNA is to anchor the substrate through this base pairing, thereby exposing the cleavage site such that cleavage is accomplished at the correct position. Our data also suggest possible base pairing between U294 in M1 RNA and the discriminator base at position +73 of the precursor. Our findings are also discussed in terms of evolution.

Key words: M1 RNA mutants/ribonuclease P/ribozyme/substrate recognition/tRNA processing

Introduction

Ribozymes are RNA molecules which function as catalysts. The best characterized ribozymes are: various derivatives of the group I introns, group II introns (Cech, 1993 and references therein), hammerhead and hairpin ribozymes (Pan *et al.*, 1993 and references therein) and RNase P RNA (Altman *et al.*, 1993). Base pairing between the substrate and the ribozyme has previously been shown to be essential for catalytic activity, with the exception of RNase P RNA.

RNase P RNA (denoted M1 RNA in *Escherichia coli*) is the catalytic subunit of RNase P, a ribonucleoprotein responsible for the maturation of the 5′-termini of tRNAs in all cell types studied so far. In the absence of the protein subunit, appropriate substrates are cleaved at the correct position by M1 RNA *in vitro* (Altman *et al.*, 1993 and references therein). The mechanism by which RNase P (or RNase P RNA) identifies its cleavage site is not known. Previous reports have suggested that RNase P recognizes the conformation of the tRNA moiety of the precursor (Altman *et al.*, 1974; Kirsebom and Altman, 1989). Furthermore, within the tRNA part, recent results have suggested that the primary structure, in particular near the cleavage site, and the length of the amino acid acceptor stem play a significant role in cleavage site selection (Altman *et al.*, 1993 and references therein; Kirsebom and Svärd, 1993; Svärd and Kirsebom, 1993). Very little is known, however, about the role that different nucleotides in M1 RNA play in this process. Previous reports have investigated possible base pairing between M1 RNA and its substrate, but no evidence demonstrating this has yet been presented (Baer *et al.*, 1988; Svärd and Kirsebom, 1993).

The CCA sequence is present in all tRNA molecules (Steinberg *et al.*, 1993). In *E.coli*, CCA is encoded in all the tRNA genes, whereas there are examples in other prokaryotes where CCA is not part of the gene, although the majority of the tRNA genes in these organisms encode CCA (Vold, 1985; Komine *et al.*, 1990 and references therein; Green and Vold, 1992, 1993). The presence of the CCA sequence is not absolutely required for cleavage by RNase P RNA *in vitro*. However, several lines of evidence suggest that this sequence is important for the rate of cleavage (Seidman and McClain, 1975; Guerrier-Takada *et al.*, 1984, 1989; Green and Vold, 1988; Svärd and Kirsebom, 1992). In addition, the presence of CCA in certain tRNA precursors is important for the location of the cleavage site (Krupp *et al.*, 1991; Kirsebom and Svärd, 1993). Furthermore, a model substrate in which the first C in CCA was changed was not cleaved by M1 RNA, suggesting that this sequence plays a crucial role in cleavage of this particular substrate (McClain *et al.*, 1987). These results encouraged us to look for nucleotides in M1 RNA which are important for the interaction with the 3′-terminus of a tRNA precursor, including CCA. Here we present evidence that G292 and G293 in M1 RNA, which are well conserved among the bacterial RNase P RNAs, base pair with the nucleotides at positions +74 and +75 (the first and second C in CCA) in the tRNA precursor. Our results also suggest possible base pairing between U294 in M1 RNA and the discriminator base at position +73 of the precursor.

Results

G293 in M1 RNA is involved in a Watson–Crick interaction with the first C in CCA

The CCA sequence at the 3′-end of tRNA molecules is an important determinant for the rate of cleavage and for the location of the cleavage site on certain tRNA precursors such as the *E.coli* $tRNA^{Ser}Su1$ precursor (pSu1) (Altman *et al.*, 1993; Kirsebom and Svärd, 1993). M1 RNA variants harbouring changes in the G291–A295 region cleave this precursor aberrantly at the −1 position (Kirsebom and Svärd, 1993). Phylogenetic studies of bacterial RNase P RNAs show that G293 and U294 in M1 RNA are well conserved (Brown and Pace, 1992). These nucleotides

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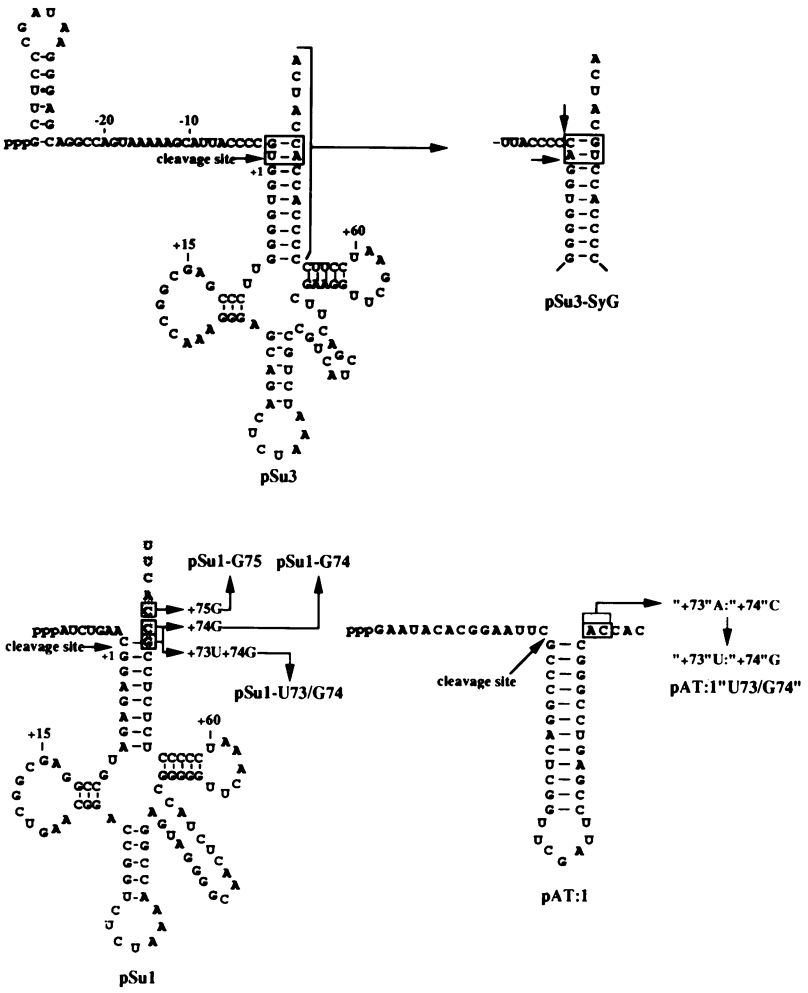

Fig. 1. Predicted secondary structures of the various precursors used in this study. The arrows indicate the RNase P cleavage sites. The boxed nucleotides are those which have been substituted in the precursor to $tRNA^{Tyr}Su3$ (pSu3) to generate pSu3-SyG, in the precursor to $tRNA^{Ser}Su1$ (pSu1) to generate pSu1-G74, pSu1-G75 and pSu1-U73/G74 and in pAT:1 to generate pAT:1'U73/G74'. The shaded nucleotides were deleted in the truncated pSu1 derivatives pSu1-Δ(−4), pSu1-Δ(−5) and pSu1-Δ(−6) (−4, −5 and −6 denote the number of nucleotides deleted from the 3′ end).

have the potential to base pair with the +73A/G (the discriminator base; McClain, 1993) and with the +74C (the first C in CCA) in the tRNA precursor. If base pairing is important for cleavage, then compensatory mutations in the ribozyme would be expected to restore proper cleavage site selection in precursors which carry changes at these positions.

To test this hypothesis we used three different sets of precursors and two M1 RNA variants (Figures 1 and 2). The precursors used were: (i) pSu1 and pSu1-74G; (ii) pSu3 and pSu3-SyG, where the latter carries substitutions at −2, −1, +73 and +74 resulting in cleavage mainly at the −2 position (Svärd and Kirsebom, 1992) (Figure 1); and (iii) pAT:1 and pAT:1'U73/G74'. The third set of precursors was chosen because cleavage of pAT:1 by wild-type M1 RNA is dependent on CCA (McClain *et al.*, 1987). Our hypothesis would predict the following. First, pSu1-74G would be cleaved aberrantly by wild-type M1 RNA, whereas a substitution of G293 by C293, which restores base pairing between the nucleotide at 293 in M1 RNA and the nucleotide at +74 in this precursor, would result in cleavage mainly at the normal position. This mutant M1 RNA is also predicted to cleave the wild-type pSu1 precursor aberrantly. Second, pSu3-SyG would be cleaved only (or mainly) at the correct position, whereas pAT:1 would be cleaved poorly by a double mutant M1 RNA carrying a C293 and an A294 that disrupt the potential base pairing ($M1_{C293A294}$ RNA; Figure 2). In contrast, the double mutant M1 RNA should cleave pAT:1'U73/G74' (where base pairing between M1 RNA and the substrate is restored) with approximately the same efficiency as wild-type M1 RNA cleaves pAT:1.

These precursors were incubated with the various M1 RNA derivatives and the results are shown in Figures 3 and 4 and summarized in Table I. A substitution of +74C by G resulted in significant cleavage at the −1 position by wild-type M1 RNA, whereas $M1_{C293}$ RNA cleaved this precursor mainly at the correct position, as predicted. As expected, this mutant M1 RNA also cleaved pSu1 at both position +1 and position −1. The presence of 5′ pCp and 5′ pGp indicates cleavage at the −1 and the +1 positions, respectively. We also showed that the 5′ cleavage fragments were of different lengths, consistent with cleavage at positions −1 and +1 (data not shown). Furthermore, the double mutant M1 RNA restored proper cleavage of pSu3-SyG and cleaved pAT:1'U73/G74' with a significantly increased efficiency compared with cleavage by wild-type M1 RNA. Note that we used four times more

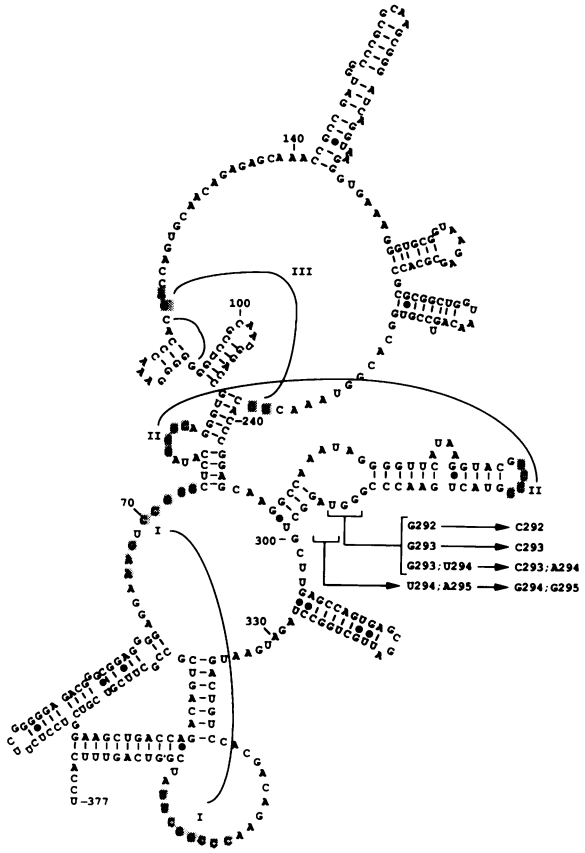

Fig. 2. The predicted secondary structure of M1 RNA according to Brown *et al.* (1993). The shaded nucleotides are involved in long range tertiary interactions, as has been described elsewhere (Brown and Pace, 1992; Tallsjö *et al.*, 1993). The mutations were introduced at the positions indicated in the Figure.

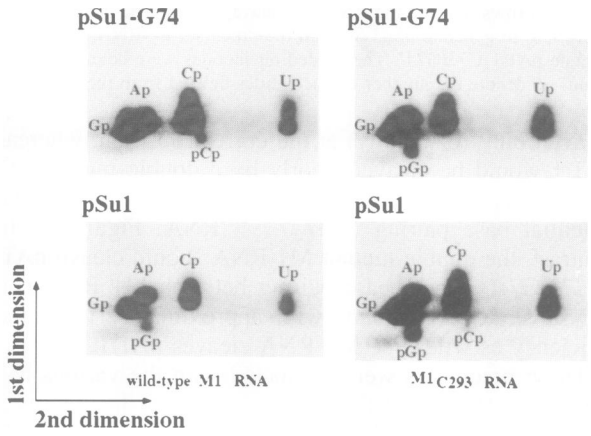

Fig. 3. Phosphoimages of two-dimensional, thin-layer chromatography illustrating the identification of 5′-termini after cleavage of pSu1 and pSu1-G74 using wild-type M1 RNA and $M1_{C293}$ RNA, as indicated. For details see Materials and methods. A 5′pGp indicates cleavage at the +1 position, whereas a 5′pCp indicates cleavage at the −1 position.

M1 RNA and several-fold longer incubation times in the absence of base pairing between M1 RNA and its substrate when cleavage of the pAT:1 derivatives was studied (Figure 4, lanes 12 and 13). In addition, we observed that pAT:1'U73/G74' was cleaved at positions −1 and +1 by wild-type M1 RNA, as indicated by the presence of two 5′ cleavage products. The 5′ matured cleavage products

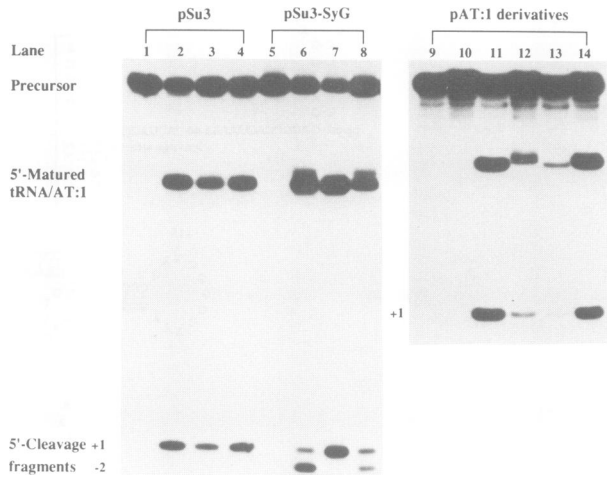

Fig. 4. Cleavage of pSu3, pSu3-SyG and the pAT:1 derivatives by wild-type M1 RNA, $M1_{C293A294}$ RNA and $M1_{G294G295}$ RNA in the absence of C5. The experiments were performed at 37°C as described in Materials and methods. The final concentrations of reactants were: precursors, 0.12 pmol/μl and M1 RNA 0.41 pmol/μl using pSu3 and pSu3-SyG; pAT:1 precursors, 0.1 pmol/μl and M1 RNA concentrations as indicated in brackets when the pAT:1 derivatives were used. The cleavage products were separated from the precursors on 10% (pSu3 and pSu3-SyG) and 20% (pAT:1 derivatives) denaturating polyacrylamide gels. Duration of cleavage is given in parentheses. Lane 1, pSu3, no enzyme added (5 min); lane 2, pSu3, wild-type M1 RNA (5 min); lane 3, pSu3, $M1_{C293A294}$ RNA (5 min); lane 4, pSu3, $M1_{G294G295}$ RNA (5 min); lane 5, pSu3-SyG, no enzyme added (60 min); lane 6, pSu3-SyG, wild-type M1 RNA (60 min); lane 7, pSu3-SyG, $M1_{C293A294}$ RNA (60 min); lane 8, pSu3-SyG, $M1_{G294G295}$ RNA (60 min); lane 9, pAT:1, no enzyme added (40 min); lane 10, pAT:1-'U73/G74', no enzyme added (40 min); lane 11, pAT:1, wild-type M1 RNA (0.02 pmol/μl) (2 min); lane 12, pAT:1'U73/G74', wild-type M1 RNA (0.082 pmol/μl) (40 min); lane 13, pAT:1, $M1_{C293A294}$ RNA (0.082 pmol/μl) (40 min); lane 14, pAT:1-'U73/G74', $M1_{C293A294}$ RNA (0.02 pmol/μl) (2 min).

derived from pAT:1 and pAT:1'U73/G74' migrated slightly differently. This was most likely due to residual secondary structure in denaturing polyacrylamide gels (Hegg and Thurlow, 1990; Svärd and Kirsebom, 1992). These results demonstrate the importance of Watson−Crick complementarity between residues G293 in M1 RNA and +74C in the precursor. We suggest that these two residues establish a base pair in the ribozyme−substrate complex.

We also tested some other M1 RNA variants carrying changes in the region G291−A294 (Kirsebom and Svärd, 1993). One of these mutants, $M1_{G294G295}$ RNA (Figure 2), cleaved pSu3-SyG at both +1 and −2 with approximately equal frequencies (Figure 4, lane 8). In contrast, a deletion of G291 and G292 (or U294 and A295) resulted in cleavage of pSu3-SyG mainly at the −2 position, as seen also with wild-type M1 RNA, demonstrating that changes at specific positions in this region of M1 RNA are required to restore cleavage site selection (see also below). The single mutant M1 RNA, $M1_{C293}$ RNA, showed a similar phenotype to $M1_{C293A294}$ RNA in cleavage of pSu3-SyG and the pAT:1 derivatives (data not shown). We infer from these results that G293 in M1 RNA base pairs with the nucleotide at position +74 in these precursors. Additionally, pSu3-SyG was cleaved with equal frequency at positions −2 and +1 by $M1_{G294G295}$ RNA, indicating that base pairing was only partially restored. This suggests the possibility that U294 is involved in base pairing with

Table I. Summary of the cleavage sites using the different tRNA precursors and various mutant M1 RNAs used in this study

Precursor	Cleavage site	M1 RNA derivative				
		Wild-type	C293	C293 A294	G294 G295	C292
pSu1	−1		8	3	34[a]	93
	+1	100	92	97	64[a]	7
pSu1-G74	−1	92	11			87
	+1	8	89			13
pSu1-Δ(−4)	−1	4				
	+1	96				
pSu1-Δ(−5)	−1	50				
	+1	50				
pSu1-Δ(−6)	−1	98				
	+1	2				
pSu1-G75	−1	94	97			18
	+1	6	3			82
pSu1-U73/G74	−1	3				
	+1	97		100		
pSu3	−2					
	+1	100	100	100	100	100
pSu3-SyG	−2	71			49	72
	+1	29	100	≥99[b]	51	28

The frequency of cleavage at the different positions is given as percentage values and was quantitated as described in Materials and methods.
[a]Data from Kirsebom and Svärd (1993).
[b]We observed some residual cleavage at the −2 position.

the nucleotide at the +73 position. The wild-type pSu3 precursor was cleaved only at the normal position, irrespective of which M1 RNA variant was used, consistent with the notion that this precursor harbours other determinants important for cleavage site selection which ensure cleavage at the correct position (Svärd and Kirsebom, 1992, 1993).

The Watson–Crick interaction also involves G292 in M1 RNA and +75C in the precursor

Cleavage of the precursor to $tRNA^{Ser}Su1$ (pSu1) in the absence of CCA occurred at position −1, demonstrating the importance of CCA for the location of the RNase P cleavage site (Kirsebom and Svärd, 1993). The observed interaction between M1 RNA and its substrate gives a rational explanation for this finding. To determine the number of nucleotides of the CCA triplet required for correct cleavage of this precursor we generated the truncated derivatives illustrated in Figure 1. The results of cleavage of these precursors are shown in Table I.

These truncated precursors were cleaved at both positions −1 and +1, suggesting that +75C and +76A are important for correct cleavage of this precursor (the latter only to a minor extent). The +75C has the potential to base pair with G292, which is strictly conserved among RNase P RNAs derived from proteobacteria (Brown *et al.*, 1991; Figure 2). To test this possibility, we changed the +75C to a G (pSu1-G75) and G292 in M1 RNA to a C ($M1_{C292}$ RNA). We predicted that this mutant precursor would be cleaved aberrantly by wild-type M1 RNA, whereas the mutant M1 RNA would restore proper cleavage site selection. In addition, the mutant would cleave the wild-type pSu1 incorrectly. The results showed that pSu1-G75 and pSu1 were cleaved mainly at the −1 position by wild-type and mutant M1 RNA, respectively,

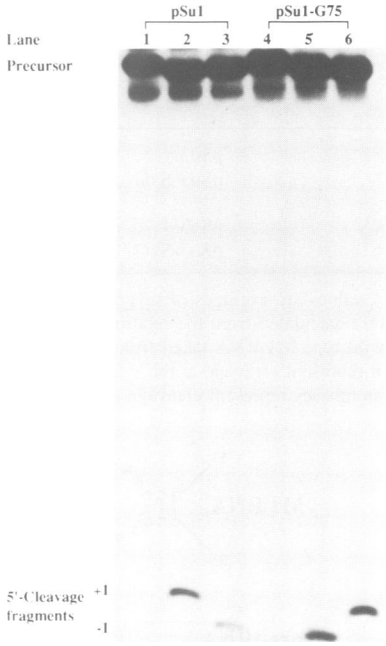

Fig. 5. Cleavage of pSu1 and pSu1-G75 by wild-type M1 RNA and $M1_{C292}$ RNA in the absence of C5. The experiments were performed at 37°C as described in Materials and methods. The final concentrations of reactants were: precursors, 0.10 pmol/μl and M1 RNA, 0.02 pmol/μl. The cleavage products were separated from the precursors on a 20% denaturating polyacrylamide gel. Duration of cleavage, 20 min. Lanes 1 and 4, pSu1 and pSu1-G75 respectively, no enzyme added; lane 2, pSu1, wild-type M1 RNA; lane 3, pSu1, $M1_{C292}$ RNA; lane 5, pSu1-G75, wild-type M1 RNA; lane 6, pSu1-G75, $M1_{C292}$ RNA.

whereas $M1_{C292}$ RNA cleaved pSu1-G75 preferentially at the correct position (Figure 5). These results are in keeping with our predictions. We therefore suggest that G292 and +75C also constitute a base pair in this ribozyme–substrate complex. The $M1_{C293}$ RNA cleaved this mutant precursor as did the wild-type M1 RNA, demonstrating specificity in the suggested base pairing between M1 RNA and its substrate (Table I).

Base pairing between M1 RNA and its substrate affects the kinetic constants

Determination of the kinetic constants for cleavage of pSu3 and pSu3-SyG by wild-type M1 RNA and $M1_{C293A294}$ RNA demonstrated a significant increase in the K_m values for those combinations in which the predicted base pairing was absent (Table II). This adds further support to the suggestion that M1 RNA base pairs with its substrate. Furthermore, the k_{cat}/K_m values can be used to express changes in transition-state stabilization energy, $\Delta\Delta G$ (Wells, 1985). These calculations show an increase in $\Delta\Delta G$ of 3–4 kcal/mol in the absence of the suggested base pairing between M1 RNA and its substrate (Table II). We also note that wild-type M1 RNA cleaved pSu3-SyG at −2 and +1 with different k_{cat}, indicating that the local structure at and near the cleavage site is important for the rate of cleavage, in particular when the 3′ end of this precursor is not involved in Watson–Crick interactions with M1 RNA.

Table II. Kinetic rate constants for cleavage of pSu3 and pSu3-SyG by wild-type M1 RNA and $M1_{C293A294}$ RNA in the absence of the C5 protein

Enzyme	Substrate	k_{cat} (min^{-1})	$K_m \times 10^8$ (M)	$k_{cat}/K_m \times 10^{-6}$ (M^{-1} min^{-1})	$\Delta\Delta G^a$ (kcal/mol)
Wild-type	pSu3	0.6[b]	1.1[b]	60[b]	
	pSu3-SyG	0.044[c]	50[c]	0.088[c]	+4.0[c]
		(0.014)[d]	(36)[d]	(0.039)[d]	(+4.5)[d]
$M1_{C293A294}$ RNA	pSu3	0.73	210	0.35	+3.2
	pSu3-SyG	1.3	2.1	62	−0.02

The k_{cat} and K_m values are averages of several independent experiments.
[a]$\Delta\Delta G$ values were calculated from the relationship $\Delta\Delta G = -RT \ln [k_{cat}/K_m]_{mut}/[k_{cat}/K_m]_{wt}$ according to Wells (1985).
[b]Numbers for wild-type M1 RNA taken from Tallsjö and Kirsebom (1993).
[c]Numbers given represent cleavage at the −2 position.
[d]Numbers in parentheses represent cleavage at the +1 position.

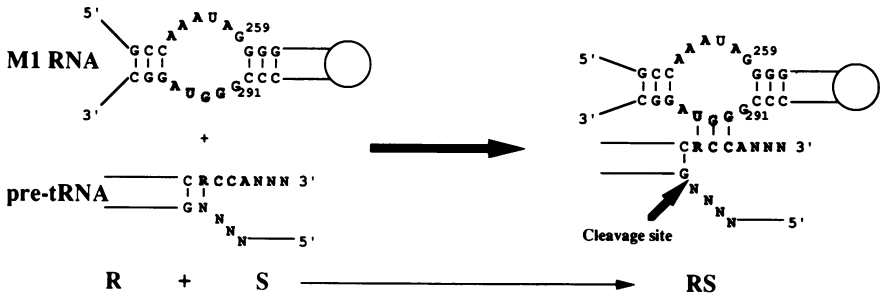

Fig. 6. Illustration of the base pairing between M1 RNA and its substrate in the ribozyme–substrate (RS) complex. The R in the precursor structure denotes a G or an A.

Discussion

Watson–Crick interaction between M1 RNA and its substrate

The 3′ end of a tRNA precursor, including the CCA sequence, is important for the rate of cleavage, as well as for the location of the cleavage site (Altman *et al.*, 1993 and references therein; Kirsebom and Svärd, 1993; this report). Here we studied cleavage site selection as well as the kinetics of cleavage of various mutants to identify nucleotides in the catalytic subunit of *E.coli* RNase P RNA (M1 RNA) which interact with this part of the precursor. By using compensatory mutations, we demonstrated the importance of Watson–Crick complementarity between two well-conserved residues in M1 RNA (G292 and G293) and two residues in the substrate (+74C and +75C, the first and second C in CCA). We suggest that these nucleotides form base pairs in the ribozyme–substrate complex (Figure 6) and that this base pairing contributes to both the catalytic activity and the cleavage site selection of the enzyme. The pSu3-SyG precursor was cleaved with equal frequencies at positions −2 and +1 by $M1_{G294G295}$ RNA, i.e. proper cleavage site selection was only partially restored. We believe that this is accomplished through base pairing between the +73U in this precursor and G294. Furthermore, the nucleotide at position +73 in the $tRNA^{His}$ precursor is a cytidine (Steinberg *et al.*, 1993) and this precursor is cleaved at the −1 position (Orellana *et al.*, 1986). The +73C cannot base pair with U294 in M1 RNA, but +74C and +75C can base pair with G292 and G293. This explains why the $tRNA^{His}$ precursor is cleaved at the −1 position. A disruption of the −1G:+73C base pair in the $tRNA^{His}$ precursor, by changing the +73C to a G, results in cleavage at both the −1 position and the +1 position. Again, this suggests possible base pairing between the +73G in this precursor and U294 in M1 RNA. This mutant $tRNA^{His}$ precursor also interacted more efficiently with M1 RNA, as indicated by the altered K_m value (Kirsebom and Svärd, 1992). Taken together, we suggest that G292, G293 and possibly also U294 base pair through Watson–Crick interactions with the nucleotides at positions +75, +74 and +73 (the discriminator base when it is a purine) respectively in the substrate. Nucleotides at these positions in M1 RNA are strictly conserved among proteobacteria (Brown *et al.*, 1991). We propose, therefore, that this base pairing applies to proteobacterial RNase P RNAs in general.

Modification–interference studies have indicated that G291 and G292 in M1 RNA are involved in the interaction with tRNA precursors (Knap *et al.*, 1990). Kazakov and Altman (1991) showed that metal ion-induced cleavage 3′ to position U294 in M1 RNA was inhibited in the presence of the precursor to $tRNA^{Tyr}Su3$. Recently it has been demonstrated that RNase P RNA is cleaved at specific positions by Pb^{2+} (Zito *et al.*, 1993; Ciesiolka *et al.*, 1994; Svärd *et al.*, 1994). Of relevance to the present study, M1 RNA was cleaved 3′ to positions 293–295. The efficiency of Pb^{2+}-induced cleavage 3′ to these positions is reduced in the presence of precursors which can base pair with nucleotides in this region of M1 RNA through the +73A, +74C and +75C. In contrast, this was not observed in the absence of such base pairing (data not shown). This finding is consistent with the results of Ciesiolka *et al.* (1994). Hartmann and co-workers have also demonstrated that the rate of Pb^{2+}-induced cleavage at the 3′ end of $tRNA^{Gly}$ is reduced in the presence of

RNase P RNA (Hardt *et al.*, 1993). The present evidence for specific Watson–Crick interaction between M1 RNA and its substrate provides a basis for interpreting these results.

Base pairing between M1 RNA and its substrate exposes the cleavage site

What does the suggested base pairing between M1 RNA and its substrate imply? Precursor tRNAs in *E.coli* usually carry both the −1/+73 and −2/+74 base pairs or just the −1/+73 base pair (Komine *et al.*, 1990 and references therein). These are disrupted as a result of the interaction with M1 RNA and as a consequence the amino acid acceptor stem of the tRNA precursor is partly unfolded. This would expose the cleavage site. Modification–interference experiments also suggest that there is unfolding of the precursor as a result of enzyme–substrate formation (Knap *et al.*, 1990). Disruption of the −1/+73 base pair as in pSu1-U73/G74 resulted in cleavage mainly at the +1 position, whereas the pSu1 derivatives pSu1-Δ(−6) and pSu1-G74, which still carry the −1/+73 base pair, were preferentially cleaved at −1 (Kirsebom and Svärd, 1993; Table I). Furthermore, a truncated derivative of the $tRNA^{Tyr}$Su3 precursor, where eight nucleotides from the 3′ end were deleted, was cleaved only at position +1 (Svärd and Kirsebom, 1992). Thus, in those precursors where the −2 and −1 nucleotides are not base paired, unfolding is not required to expose the cleavage site and consequently base pairing between M1 RNA and its substrate is not crucial for cleavage at the correct position. In this context we emphasize that cleavage site selection on various tRNA precursors is dependent on several determinants and changing or deleting one does not necessarily affect the location of the cleavage site. This has been discussed in detail elsewhere (Svärd and Kirsebom, 1992, 1993).

It has been demonstrated that Mg^{2+} spontaneously cleaves M1 RNA at several positions, e.g. 3′ to position 294. In addition, Mg^{2+} cleaved the $tRNA^{Tyr}$Su3 precursor between positions −2 and −3. These results suggest that Mg^{2+} binds in the vicinity of these positions in M1 RNA and in this precursor (Kazakov and Altman, 1991). In addition, Perreault and Altman (1992) showed that the 2′-OH at positions near the cleavage site in a model substrate are important for binding of Mg^{2+}. Changes in the region 291–295 in M1 RNA resulted in a significant reduction in the rate of cleavage by Mg^{2+} between positions 294 and 295 (Kirsebom and Svärd, 1993). We suggest that the Mg^{2+} ions which are bound in the vicinity of the −2 position in the precursor and the 294 position in M1 RNA are re-coordinated as a result of base pairing between the substrate and M1 RNA. As a consequence, cleavage at the correct position is accomplished.

In conclusion, we suggest that a function of M1 RNA is to anchor the substrate through base pairing, thereby exposing the cleavage site and influencing cleavage site selection through a re-coordination of Mg^{2+} ion(s). Thus, base pairing is an important feature of M1 RNA activity, as previously demonstrated for other ribozymes (Cech, 1993; Pan *et al.*, 1993). This finding will allow us to manipulate the interaction between M1 RNA and its substrate in a predictable manner. The suggested base pairing in the ribozyme–substrate complex also indicates that G292, G293 and U294 in particular are part of (or are very close to) the active centre of M1 RNA, assuming that the rest of the amino acid acceptor stem of the precursor remains folded. Here we also note that disruption of base pairing as a result of enzyme–substrate complex formation has been observed in the cognate $tRNA^{Gln}$–glutaminyl-tRNA synthetase complex (Rould *et al.*, 1989).

Evolutionary implications

Certain specific G and U residues in domain V in *E.coli* 23S rRNA are protected by oligonucleotide fragments carrying CCA (Moazed and Noller, 1991). Recently, it was shown that mutations at positions G2252 and G2253, which are protected by CCA, result in mutants which affect the accuracy of translation (Gregory *et al.*, 1994). These findings might indicate further similarities in the mechanisms through which M1 RNA and 23S rRNA interact with their respective substrates (see also Guerrier-Takada *et al.*, 1989). It remains to be seen whether G2252 and G2253 in 23S rRNA base pair with the two Cs in CCA. The genomic tag hypothesis suggests that a 3′ terminal CCA base paired with the sequence 5′-GG may have been selected to function in the initiation of replication of RNA molecules (Maizels and Weiner, 1993). The base pairing presented here and the specific interaction between CCA and 23S rRNA could conceivably be ancient remnants of an RNA world.

Materials and methods

Plasmid constructions and generation of tRNA precursors and mutant M1 RNA molecules

The construction of genes encoding precursors to $tRNA^{Ser}$Su1 [pSu1 and pSu1-Δ(−6)], $tRNA^{Tyr}$Su3 (pSu3) and $tRNA^{Tyr}$Su3-SyG (pSu3-SyG) behind the T7 promoter has been described elsewhere (Kirsebom and Svärd, 1992, 1993; Svärd and Kirsebom, 1992). The base substituted variant of pSu1 used in this study was constructed using the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) where one of the oligonucleotides carried the changes necessary to generate the desired mutant precursor gene. The PCR amplified gene(s) was blunt-end ligated into pUC19 which had been cut with *Sma*I.

The pAT:1 and pAT:1'U73/G74' genes behind the T7 promoter were synthesized on an Applied Biosystems 394 DNA-RNA Synthesizer.

The construction of the wild-type M1 RNA and $M1_{G294G295}$ RNA genes behind the T7 promoter has been described elsewhere (Vioque *et al.*, 1988; Kirsebom and Svärd, 1993). The C292, C293 and C293/A294 substitutions were introduced using the Pharmacia Biotech USE Mutagenesis Kit according to the manufacturer's instructions, using pJA2 as template (Vioque *et al.*, 1988), or by PCR as described elsewhere (Kirsebom and Svärd, 1993).

The different gene constructs were verified by DNA sequencing according to Sanger *et al.* (1977).

The precursor genes carried appropriate restriction sites to generate the desired precursor 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 described in detail elsewhere (Guerrier-Takada *et al.*, 1983, 1988; Vioque *et al.*, 1988; Kirsebom and Svärd, 1992).

Determinations of the kinetic constants were performed at 37°C as described elsewhere (Kirsebom and Svärd, 1992), except that the 5′ cleavage fragment was used for the calculations instead of the 5′ matured tRNA.

Verification and quantification of the cleavage site

The cleavage site on the pSu1 derivatives was verified by thin-layer chromatography according to Nishimura (1972), as described elsewhere (Kirsebom and Svärd, 1992).

The frequency of cleavage at different positions was quantitated from the relative amount of 5′ cleavage products of precursors which were cleaved at more than one position, except for cleavage of pSu1-U73/G74. The relative amount of 5′ cleavage products was quantitated using a PhosphorImager 400S (Molecular Dynamics). The frequencies of cleavage at positions −1 and +1 on pSu1-U73/G74 were quantitated according to Kirsebom and Svärd (1992).

Acknowledgements

This paper is dedicated to the memory of our friend T.Ruusala. We thank Drs E.Bridge, S.Burnett, M.Ehrenberg and A.Virtanen for comments on the manuscript. This work was supported by grants to L.A.K. from the Swedish Natural Science Research Council and the Swedish Research Council for Engineering Sciences.

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Received on June 7, 1994; revised on July 27, 1994