The EMBO Journal vol.14 no.12 pp.2935–2944, 1995

Rp-phosphorothioate modifications in RNase P RNA that interfere with tRNA binding

Wolf-Dietrich Hardt, Jens M.Warnecke, Volker A.Erdmann and Roland K.Hartmann[1]

Institut für Biochemie, Freie Universität Berlin, Thielallee 63, 14195 Berlin, Germany

[1]Corresponding author

Communicated by V.A.Erdmann

We have used Rp-phosphorothioate modifications and a binding interference assay to analyse the role of phosphate oxygens in tRNA recognition by *Escherichia coli* ribonuclease P (RNase P) RNA. Total (100%) Rp-phosphorothioate modification at A, C or G positions of RNase P RNA strongly impaired tRNA binding and pre-tRNA processing, while effects were less pronounced at U positions. Partially modified *E.coli* RNase P RNAs were separated into tRNA binding and non-binding fractions by gel retardation. Rp-phosphorothioate modifications that interfered with tRNA binding were found 5′ of nucleotides A_{67}, G_{68}, U_{69}, C_{70}, C_{71}, G_{72}, A_{130}, A_{132}, A_{248}, A_{249}, G_{300}, A_{317}, A_{330}, A_{352}, C_{353} and C_{354}. Manganese rescue at positions U_{69}, C_{70}, A_{130} and A_{132} identified, for the first time, sites of direct metal ion coordination in RNase P RNA. Most sites of interference are at strongly conserved nucleotides and nine reside within a long-range base-pairing interaction present in all known RNase P RNAs. In contrast to RNase P RNA, 100% Rp-phosphorothioate substitutions in tRNA showed only moderate effects on binding to RNase P RNAs from *E.coli, Bacillus subtilis* and *Chromatium vinosum*, suggesting that *pro*-Rp phosphate oxygens of mature tRNA contribute relatively little to the formation of the tRNA–RNase P RNA complex.

Key words: manganese rescue/RNase P/Rp-phosphorothioate modifications/tRNA binding

Introduction

Ribonuclease P (RNase P) is an essential structure-specific endoribonuclease that generates the mature 5′ ends of tRNAs. All RNase P enzymes analysed so far, except for the activity from spinach chloroplasts (Wang *et al.*, 1988), were found to be ribonucleoprotein particles. RNA subunits of RNase P enzymes from bacteria are catalytically active *in vitro* in the absence of protein components (Guerrier-Takada *et al.*, 1983). They are the only known RNA catalysts naturally devoted to act *in trans*.

RNase P enzymes recognize their substrates mainly by tertiary structural features common to the mature domains of all tRNAs. Recognition elements in tRNAs have been investigated extensively by site-directed mutagenesis (Leontis *et al.*, 1988; Nichols *et al.*, 1988; Drainas *et al.*, 1989; Svärd and Kirsebom, 1992, 1993; Yuan *et al.*, 1992; Altman *et al.*, 1993, and references therein; Kirsebom and Svärd, 1993, 1994; Hardt *et al.*, 1995) and deletion analyses (McClain *et al.*, 1987; Forster and Altman, 1990a; Schlegl *et al.*, 1992; Hardt *et al.*, 1993a; Yuan and Altman, 1995), modification interference studies (Kahle *et al.*, 1990; Thurlow *et al.*, 1991), cross-linking and chemical probing studies (Guerrier-Takada *et al.*, 1989; Knap *et al.*, 1990; Gaur and Krupp, 1993; Harris *et al.*, 1994, and references therein; Oh and Pace, 1994). For RNase P enzymes from bacteria, a picture has emerged in which co-axially stacked T-arm and acceptor stem harbour the major recognition elements. The D-arm seems to support recognition by indirect means, whereas the anticodon arm is dispensable for substrate recognition (Hardt *et al.* 1993a). The conserved CCA 3′-end is an important recognition feature in reactions catalysed by RNase P RNA subunits (Guerrier-Takada *et al.*, 1984, 1989; McClain *et al.*, 1987; Green and Vold, 1988; Svärd and Kirsebom, 1992; Kirsebom and Svärd, 1993, 1994; Oh and Pace, 1994; Hardt *et al.*, 1995). Nucleotides –1 to –4 of the precursor segment of pre-tRNAs may form additional contacts with bacterial RNase P RNA (Svärd and Kirsebom, 1992, 1993; Altman *et al.*, 1993, and references therein; Kirsebom and Svärd, 1993; LaGrandeur *et al.*, 1994).

Two models of the *Escherichia coli* RNase P RNA–pre-tRNA complex were published recently (Harris *et al.*, 1994; Westhof and Altman, 1994). Distance constraints obtained by a number of photocross-links between derivatives of *Bacillus subtilis* $tRNA^{Asp}$ and *E.coli* RNase P RNA provided a major contribution to these models. However, due to the lack of suitable assay systems only very limited data are available, so far, concerning functional groups of RNase P RNA that contribute to the higher order structure of the ribozyme and its specific interaction with tRNA molecules.

In the study presented here, we have investigated the role of *pro*-Rp oxygens in formation of the tRNA–RNase P RNA complex. Rp-phosphorothioates, in which one non-bridging phosphate oxygen is replaced with sulfur, are thought to interfere with hydrogen bonding and Mg^{2+} coordination, while leaving the secondary structure largely unaffected (Christian and Yarus, 1992, 1993 and references therein). A recently developed gel retardation system (Hardt *et al.*, 1993b) allows separation of RNase P RNA–tRNA complexes from unbound tRNA and RNase P RNA. This assay was employed to analyse 100% Rp-phosphorothioate-modified RNase P RNAs and tRNAs and to screen for distinct Rp-phosphorothioate positions in partially modified RNase P RNA that interfere with tRNA binding. We have identified 16 phosphorothioate modifications that impair tRNA binding, most of which reside in conserved regions of RNase P RNA. Remarkably,

© Oxford University Press

nine of these 16 positions are in the region of a long-range base-pairing interaction, which is conserved in all RNase P RNA subunits known so far and which is also present in the RNA subunit of RNase MRP (Forster and Altman, 1990b; Schmitt *et al.*, 1993). Mn^{2+} rescue relieved phosphorothioate interferences at four of the 16 positions, suggesting that *pro*-Rp oxygens at these locations are directly involved in the coordination of divalent metal ions. Phylogenetic and biochemical evidence suggests that at least some of the positions of the *E.coli* RNase P RNA backbone, for which we have detected phosphorothioate interference with tRNA binding, are located at the interface between tRNA and RNase P RNA. At other positions, sulfur substitutions may perturb the higher order structure of RNase P RNA. Locations of interfering positions are discussed in the context of the current three-dimensional models of the *E.coli* RNase P RNA–pre-tRNA complex (Harris *et al.*, 1994; Westhof and Altman, 1994). Most are clustered in the RNase P RNA core, in proximity to the scissile phosphodiester bond in pre-tRNA. Positions of interference are somewhat more dispersed in the model by Westhof and Altman (1994). Nevertheless, A_{330}, A_{352}, C_{353} and C_{354} are close to the 5′-end of the mature tRNA moiety in both models.

Results

Fully (100%) Rp-phosphorothioate-modified E.coli RNase P RNA

Fully (100%) modified *E.coli* RNase P RNAs were obtained by transcription with T7 RNA polymerase. NTPs were replaced by Sp-NTPαS analogues, yielding Rp-phosphorothioate-modified RNAs due to inversion of configuration at the phosphorus atom during polymerization (Griffiths *et al.*, 1987). Processing of the pre-$tRNA^{Gly}$ from *Thermus thermophilus* (Schlegl *et al.*, 1992) was virtually abolished in the presence of RNase P RNAs that were 100% modified at A, C or G positions, as inferred from single turnover kinetics with excess amounts of RNase P RNA (Figure 1a). Complete modification at U positions reduced the rate of pre-$tRNA^{Gly}$ cleavage 10-fold compared with the unmodified *E.coli* RNase P RNA.

A gel retardation system (Hardt *et al.*, 1993b) was used to test the fully modified RNase P RNAs for tRNA binding under *in vitro* cleavage assay buffer conditions. Complete substitution at U residues led to 10-fold weaker binding of ^{32}P-labelled $tRNA^{Gly}$ from *T.thermophilus*, while no RNase P RNA–tRNA complexes were observed with *E.coli* RNase P RNA modified at A, C or G positions (Figure 1b). These data showed clearly that phosphorothioate modification of the sugar–phosphate backbone of RNase P RNA interferes with both maturation of pre-tRNA and tRNA binding.

tRNA binding by partially modified E.coli RNase P RNA

The physical separation of the RNase P RNA–tRNA complex from free RNase P RNA and free tRNA (Hardt *et al.*, 1993b) allowed to select for tRNA binding under standard *in vitro* cleavage assay conditions. To enhance resolution we have increased the size of the tRNA by implanting the 60 nucleotide intron of pre-$tRNA^{Ile}$ from

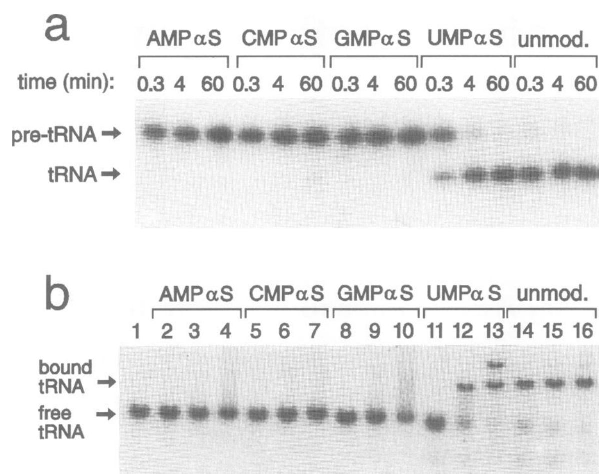

Fig. 1. Kinetic performance and tRNA binding affinity of 100% Rp-phosphorothioate-modified *E.coli* RNase P RNAs. (**a**) Trace amounts (<0.005 μM) of ^{32}P-labelled pre-$tRNA^{Gly}$ were cleaved in the presence of 3 μM 100% modified *E.coli* RNase P RNAs in buffer A at 37°C. Aliquots were withdrawn at the indicated times and analysed on a 9% polyacrylamide–8 M urea gel. (**b**) Trace amounts (<0.005 μM) of ^{32}P-labelled pre-RNA^{Gly} were incubated with 100% modified *E.coli* RNase P RNAs in buffer A containing 5% glycerol and analysed on a native polyacrylamide gel equilibrated with the same buffer (see Materials and methods). Lane 1, no RNase P RNA added; lanes 2, 5, 8, 11 and 14: 0.03 μM RNase P RNA; lanes 3, 6, 9, 12 and 15: 0.3 μM RNase P RNA; lanes 4, 7, 10, 13 and 16: 3 μM RNase P RNA.

Saccharomyces cerevisiae into the anticodon loop of $tRNA^{Gly}$ from *T.thermophilus*. This tRNA will be referred to as $tRNA^{Gly}_{(intron)}$ throughout this article. The $tRNA^{Gly}_{(intron)}$ and $tRNA^{Gly}$ showed no significant differences in binding to RNase P RNA: (i) binding of $tRNA^{Gly}$ and $tRNA^{Gly}_{(intron)}$ is mutually exclusive, i.e. both tRNAs compete for the same binding site on RNase P RNA, (ii) processing of pre-$tRNA^{Gly}$ is inhibited to the same extent by excess amounts of $tRNA^{Gly}$ and $tRNA^{Gly}_{(intron)}$, (iii) complex formation was strongly impaired for $tRNA^{Gly}$ as well as for the $tRNA^{Gly}_{(intron)}$ when nucleotide +1 was deleted or when the CCA terminus was changed to GCA in the two RNAs (Hardt *et al.*, 1993b, 1995). In conclusion, we consider the gel retardation assay using $tRNA^{Gly}_{(intron)}$ a sensitive tool to screen pools of randomly modified RNase P RNAs for those chemical modifications that interfere with the formation of the specific tRNA–RNase P RNA complex.

In initial experiments, we have analysed tRNA binding of RNase P RNAs with different extents of modification which were obtained by varying the proportions of NTP and Sp-NTPαS during T7 transcription. Incorporation of NMPαS analogues was shown to correspond closely to the relative concentrations of NTPαS and NTP in transcription assays (Christian and Yarus, 1992). At 0.7 μM $tRNA^{Gly}_{(intron)}$, increasing degrees of Rp-phosphorothioate substitution of the *E.coli* RNase P RNA (0.4 μM) reduced the fraction of RNase P RNA that formed a specific complex with the $tRNA^{Gly}_{(intron)}$. T7 transcription including 5% of either Sp-ATPαS, Sp-GTPαS, Sp-UTPαS or 10% of Sp-CTPαS (see Materials and methods) yielded RNase P RNA pools with a somewhat reduced capability of $tRNA^{Gly}_{(intron)}$ binding (data not shown). These pools were chosen for subsequent analyses. A proportion of 5%

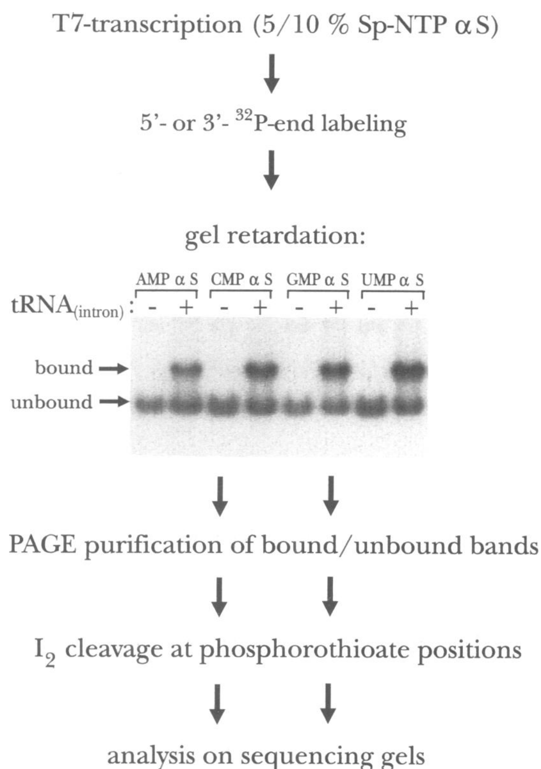

Fig. 2. Flow scheme of the phosphorothioate binding interference assay. T7 transcription in the presence of small amounts (5 or 10%) of Sp-NTPαS yields *E.coli* RNase P RNAs which are partially modified with Rp-phosphorothioates 5′ of either A, C, G or U residues. ^{32}P end-labelled RNase P RNA (1.2 μM) was separated into binding and non-binding fractions by gel retardation in the presence (+) of unlabelled $tRNA^{Gly}_{(intron)}$ (1.9 μM); (–), control lanes in the absence of $tRNA^{Gly}_{(intron)}$. Bands corresponding to free RNase P RNA and RNase P RNA bound to $tRNA^{Gly}_{(intron)}$ were excised from the gel and further purified on 5% polyacrylamide–8 M urea gels. Bands containing the intact RNase P RNA were excised from the gel, eluted and recovered by ethanol precipitation. The material was subjected to iodine cleavage, desalted by ethanol precipitation and analysed on 5, 6, 8 and 10% polyacrylamide–8 M urea gels.

ATPαS, GTPαS or UTPαS or 10% CTPαS during transcription would imply that an average *E.coli* RNase P RNA molecule had either three or four of its 87 As, two or three of its 57 Us, six to nine of its 133 Gs or 10 of its 101 Cs modified. Poisson distribution predicts that >95% of the modified molecules contain only one of the identified functionally important phosphorothioate substitutions.

Certain Rp-phosphorothioate positions interfere with tRNA binding

The binding interference protocol is outlined in Figure 2. In the final step, the iodine cleavage pattern of the non-binding RNase P RNA fraction was compared with that of RNase P RNA derived from the complex with $tRNA^{Gly}_{(intron)}$ (Figure 3). At positions where Rp-phosphorothioates interfered with gel-resolvable binding of $tRNA^{Gly}_{(intron)}$, the intensity of the corresponding iodine cleavage band was reduced in RNase P RNA recovered from the complex (Figure 3, lanes 3), while the intensity of the same iodine cleavage band was enhanced in lanes corresponding to the non-binding RNase P RNA fraction (Figure 3, lanes 4). The original RNase P RNA (Figure 3, lanes 1) and RNase P RNA that went through the whole procedure without selection for $tRNA^{Gly}_{(intron)}$ binding (Figure 3, lanes 2) served as the controls.

Autoradiographs from at least six independent experiments revealed, in a highly reproducible manner, strong interference with gel-resolvable $tRNA^{Gly}_{(intron)}$ binding for Rp-phosphorothioates 5′ of positions A_{67}, G_{68}, C_{70}, A_{130}, A_{132}, A_{248}, A_{352}, C_{353} and C_{354}, and a moderate interference (~50% reduced intensity) at U_{69}, C_{71}, A_{249}, G_{300}, A_{317} and A_{330}. A phosphorothioate 5′ of G_{72} resulted in weak (~20%) but highly reproducible interference. Interference at a few positions could not be interpreted unambiguously. For example, we found reproducible phosphorothioate interference at position C_{131} (Figure 3, lanes 3 and 4, CMPαS). However, we frequently observed a band at the same position in untreated control lanes (Figure 3, lanes 5–8, CMPαS; Figure 4, lanes 6 and 7, CMPαS), suggesting that the iodine cleavage pattern might have been obscured by this unspecific degradation. For this reason, position C_{131} has been excluded from the list of interfering positions.

We did not detect iodine cleavage bands of enhanced intensity in the binding-competent RNase P RNA, which would have indicated sulfur substitutions that increase binding affinity for the $tRNA^{Gly}_{(intron)}$.

To identify positions of interference that cause only minor reductions in binding affinity, we performed binding interference experiments in the presence of ~10-fold higher concentrations of $tRNA^{Gly}_{(intron)}$ (16 μM) and RNase P RNA (13 μM). A simultaneous increase in the concentration of both RNAs still allowed almost equal amounts of binding and non-binding RNase P RNA to be obtained. Under these conditions, phosphorothioate interference was alleviated at positions A_{67}, G_{68}, C_{70}, C_{71}, A_{130} and A_{132} (data not shown).

Manganese rescue

The involvement of *pro*-Rp oxygens in metal ion coordination was analysed by manganese rescue. It is known that Mn^{2+} can replace Mg^{2+} in the RNase P RNA cleavage reaction (Smith *et al.*, 1992). In addition, tRNA binding affinity measured by gel retardation was unchanged (data not shown) in the presence of 5 mM Mn^{2+}/95 mM Mg^{2+} (buffer B) instead of 100 mM Mg^{2+} (buffer A). The detection of phosphorothioates that interfere with metal ion binding is based on a relatively small effect of sulfur substitutions on the coordination to Mn^{2+} compared with a dramatic effect on the coordination to Mg^{2+} (Jaffe and Cohn, 1979; Pecoraro *et al.*, 1984). Therefore, partial substitutions of Mg^{2+} by Mn^{2+} may, at least partially, suppress phosphorothioate interference at positions that are involved in the direct coordination of functionally important divalent metal ions (Christian and Yarus, 1993). A partial, instead of complete, substitution of Mn^{2+} for Mg^{2+} is likely to minimize perturbations of RNA structure which are expected for the presence of Mn^{2+} at all metal binding sites due to its distinct coordination properties (Jack *et al.*, 1977). Mn^{2+}-specific structural distortion has been described for position G_{201} of the *Tetrahymena* intron in a phosphorothioate interference study of the G-addition reaction (Christian and Yarus, 1993).

Gel separation of binding from non-binding *E.coli* RNase P RNAs in the presence of 5 mM Mn^{2+}/95

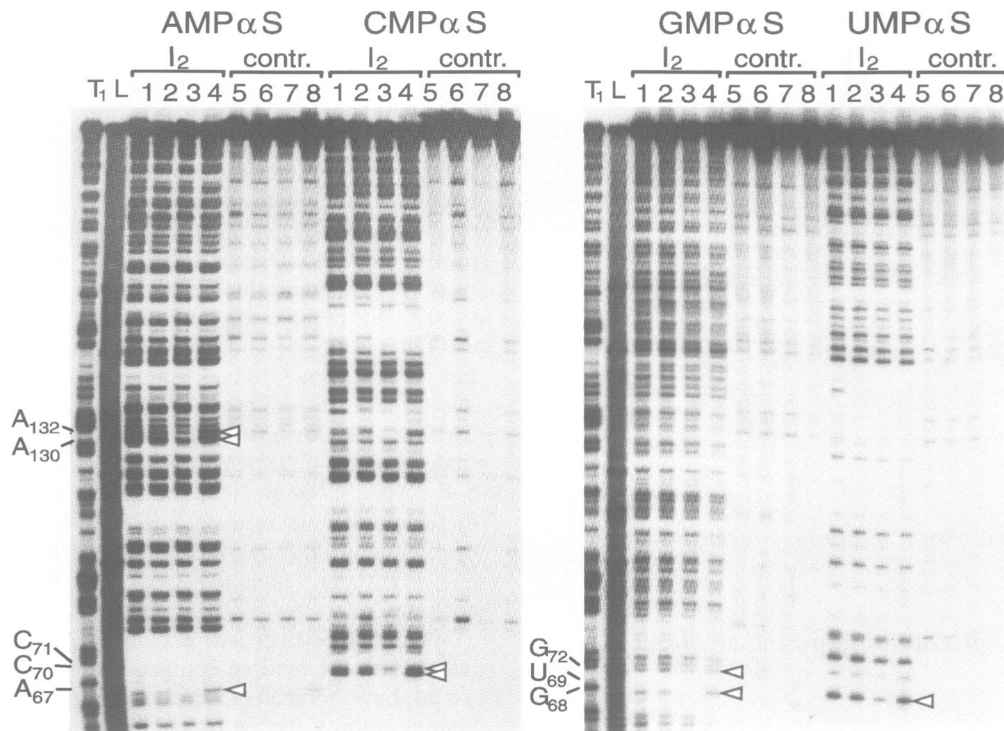

Fig. 3. Iodine cleavage analysis of a phosphorothioate binding-interference experiment with partially phosphorothioate modified 5′-^{32}P-labelled *E.coli* RNase P RNAs selected for $tRNA^{Gly}_{(intron)}$ binding under conditions of buffer A (100 mM Mg^{2+}). Lanes 1, iodine cleavage pattern of the original material; lanes 2, iodine cleavage pattern of RNase P RNAs that went through the whole procedure without selection for $tRNA^{Gly}_{(intron)}$ binding, as outlined in Figure 2 legend; lanes 3, iodine cleavage pattern of RNase P RNA recovered from the complex with $tRNA^{Gly}_{(intron)}$; lanes 4, iodine cleavage pattern of the RNase P RNA fraction that did not bind to $tRNA^{Gly}_{(intron)}$; lanes 5–8 correspond with lanes 1–4 except that the iodine cleavage step was omitted. Lanes T_1, limited digestion with RNase T_1; L, limited alkaline hydrolysis. Some nucleotide positions of *E.coli* RNase P RNA are indicated next to lanes T_1. Open arrowheads mark the positions of phosphorothioate interference.

mM Mg^{2+} relieved interference of Rp-phosphorothioate modifications at positions U_{69}, C_{70}, A_{130} and A_{132} (Figure 4). This suggests that Mg^{2+} ions binding to the corresponding *pro*-Rp oxygens directly or indirectly support tRNA binding. No other Mn^{2+}-specific effects were observed (Figure 4; compare lanes 1 with 3 and 2 with 4; data for GMPαS and UMPαS modifications not shown).

Modification of tRNA

In a complementary approach, we studied the effects of Rp-phosphorothioate modifications in tRNA on binding to RNase P RNAs. Fully modified mature $tRNA^{Gly}$ from *T.thermophilus* and $tRNA^{Phe}$ from *S.cerevisiae* (Sampson and Uhlenbeck, 1988) carried Rp-phosphorothioates 5′ of either A, C, U or G residues (except for the 5′-terminal G, since T7 transcripts were initiated with GMP, see Materials and methods). The tRNAs were labelled internally by addition of either [α-^{32}P]ATP or [α-^{32}P]CTP during transcription. Binding to unlabelled RNase P RNAs from *E.coli*, *B.subtilis* and *Chromatium vinosum* was measured by gel retardation. Phosphorothioate modifications 5′ of either A, G or U positions showed only minor effects on RNase P RNA binding affinity (not exceeding a 2- to 3-fold reduction compared with unmodified tRNA), no matter which RNase P RNA was used (data not shown). Rp-phosphorothioate modifications 5′ of the C residues had the most pronounced effect. However, apparent dissociation constants were raised not more than 10-fold compared with unmodified tRNA. The same results were obtained when we used conditions of 95 mM Mg^{2+} and 5 mM Mn^{2+} (data not shown).

Discussion

So far, the processing interference approach, easily applicable to self-splicing introns and *cis*-acting ribozymes, has not been feasible for RNase P since the enzyme catalyses tRNA maturation *in trans*. One solution to this problem may be the use of self-cleaving tRNA–RNase P RNA conjugates, although end-to-end conjugates may have a lower catalytic efficiency (Kikuchi *et al.*, 1993; Frank *et al.*, 1994). Constructs, in which the pre-tRNA is tethered to internal sites of circularly permuted RNase P RNAs, may be most appropriate for cleavage interference studies, although largely increased concentrations of monovalent cations and elevated incubation temperatures were shown to be required for efficient processing (Frank *et al.*, 1994).

The study presented here, which monitors binding of mature tRNA apart from catalysis, is suited to detect modifications of the *E.coli* RNase P RNA backbone that interfere with formation of the specific RNase P RNA–tRNA complex. Since release of matured tRNA from *E.coli* RNase P RNA is rate-limiting in multiple turnover reactions (Reich *et al.*, 1988; Tallsjö and Kirsebom, 1993), modifications that interfere with tRNA binding are at least relevant to this step of the catalytic cycle.

Although this study focuses on the binding of mature tRNA to *E.coli* RNase P RNA, several lines of evidence

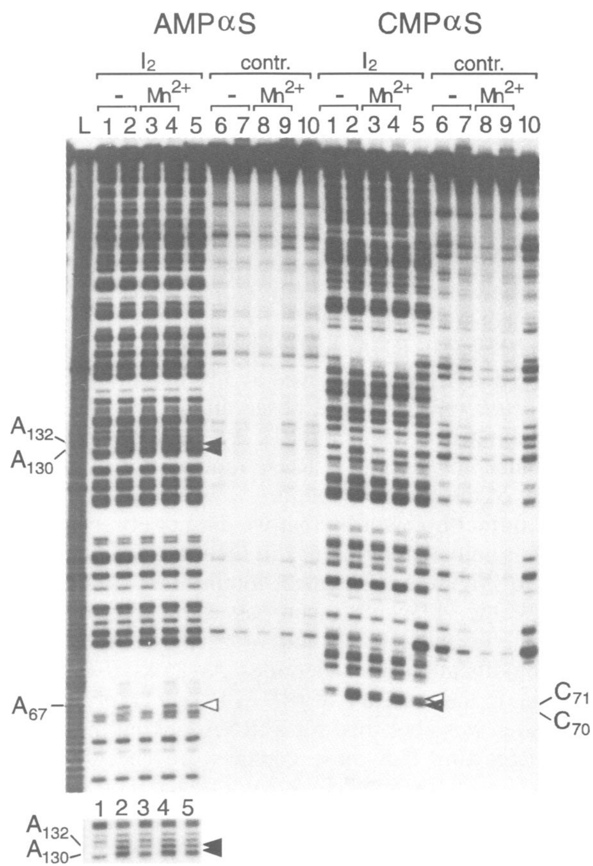

Fig. 4. Iodine cleavage analysis of a manganese rescue experiment using partially phosphorothioate-modified 5′-^{32}P-labelled *E.coli* RNase P RNAs. Lanes 1, RNase P RNA recovered from the complex with $tRNA^{Gly}_{(intron)}$ under conditions of buffer A (100 mM Mg^{2+}); lanes 2, RNase P RNA derived from the fraction that did not bind to $tRNA^{Gly}_{(intron)}$ under conditions of buffer A; lanes 3 and 4, as lanes 1 and 2, respectively, but selected for $tRNA^{Gly}_{(intron)}$ binding under conditions of buffer B (5 mM Mn^{2+} and 95 mM Mg^{2+}); lanes 5, iodine cleavage pattern of the original material. Lanes 6–10 correspond with lanes 1–5 except that the iodine cleavage was omitted; lane L, limited alkaline hydrolysis. Some nucleotide positions of *E.coli* RNase P RNA are indicated next to lanes L and 10. Solid arrowheads mark positions of manganese rescue, and open arrowheads indicate positions of phosphorothioate interference which were not affected by manganese under the conditions tested. For lanes 1–5, a weaker film exposure of the region comprising A_{130} and A_{132} is shown at the bottom.

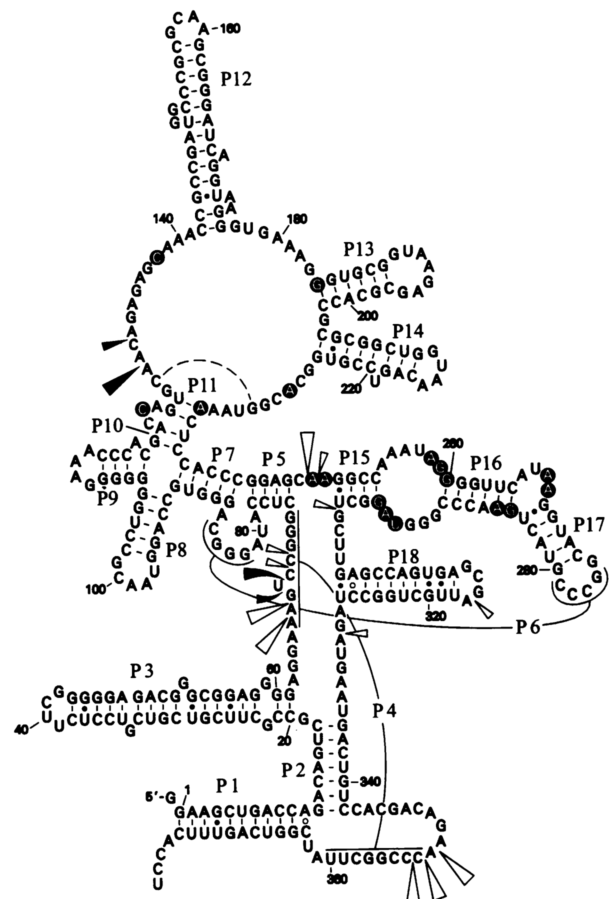

Fig. 5. Secondary structure of *E.coli* RNase P RNA according to Haas *et al.*, 1994. Positions which showed Rp-phosphorothioate interference in the binding assay are marked by arrows; large and small arrowheads indicate that >50% (large arrowheads) or ≤50% (small arrowheads) of the radioactivity was lost from the corresponding band in the iodine cleavage pattern of the binding-competent RNase P RNA fraction. Solid arrowheads indicate phosphorothioate interferences that could be rescued by manganese. The broken line marks a novel tertiary interaction identified recently (Mattsson *et al.*, 1994). Specific sites of Pb^{2+}-induced hydrolysis were assigned to phosphodiester bonds 5′ of the highlighted nucleotides (Ciesiolka *et al.*, 1994); hydrolysis at A_{234}, A_{258}–G_{260} and U_{294}–G_{296} was largely reduced due to binding of tRNA, whereas hydrolysis at G_{285}/A_{286} occurred only when *E.coli* RNase P RNA was complexed with tRNA.

indicate that pre-tRNA and mature tRNA share at least one strongly overlapping or even identical mode of binding. Thus, data obtained for binding of mature tRNA are likely to be also relevant to binding of the pre-tRNA substrate. For example, mature tRNA was a purely competitive inhibitor in the pre-tRNA cleavage reaction of RNase P RNA (Smith *et al.*, 1992), indicating that precursor and mature tRNA occupy the same binding site. Likewise, K_i values of mature tRNA were very similar to K_m values in pre-tRNA cleavage reactions (Smith *et al.*, 1992; Tallsjö & Kirsebom, 1993), suggesting that precursor and mature tRNAs bind with similar affinity and kinetics to RNase P RNAs. Also, cross-linking data obtained with pre-tRNA and mature tRNA carrying a photoagent at the 3′-end (Oh and Pace, 1994) as well as chemical modification of RNase P RNA in the presence of pre-tRNA and mature tRNA (LaGrandeur *et al.*, 1994) gave very similar results.

Sites of interference

Strong interferences were found for Rp-phosphorothioates 5′ of A_{67}, G_{68}, C_{70}, A_{130}, A_{132}, A_{248}, A_{352}, C_{353} and C_{354}, moderate interference 5′ of positions U_{69}, C_{71}, A_{249}, G_{300}, A_{317} and A_{330} and a very weak but highly reproducible interference at position G_{72} (Figures 3 and 5).

When binding selection was performed at ~10-fold higher RNA concentrations, phosphorothioate interference was decreased at positions A_{67}, G_{68}, C_{70}, C_{71}, A_{130} and A_{132}, indicating that these modifications only slightly destabilize the tRNA–RNase P RNA complex. Note that a loss of 2 kcal/mol of binding energy corresponds to a >20-fold increase in the apparent equilibrium binding constant. In principle, interferences might result from more than one tRNA binding mode of similar affinity. Thus, it cannot be excluded that decreased interferences at positions A_{67}, G_{68}, C_{70}, C_{71}, A_{130} and A_{132} in the presence of higher RNA concentrations might be attributable to

one tRNA binding mode, and those remaining unaffected by the RNA concentration to another. Westhof and Altman (1994) interpreted the bulk of cross-linking and structural probing data to indicate the existence of more than one (pre-)tRNA binding mode.

Moderate interferences at U_{69}, G_{300}, A_{317} and A_{330} and strong interferences at A_{352}, C_{353} and C_{354} were not affected by increasing RNA concentrations. This may suggest that these modifications trap a fraction of RNase P RNA molecules in a binding-deficient conformation or that major single contacts with the tRNA are disrupted to such an extent that tRNA binding is disfavoured, even at elevated RNA concentrations.

Most of the identified positions coincide with base identities that are either highly (A_{67}, A_{130}) or universally conserved in bacteria (G_{68}, U_{69}, C_{70}, C_{71}, A_{132}, A_{248}, A_{249}, G_{300}, A_{330}; Haas *et al.*, 1994). Nucleotides U_{69}, C_{70} and A_{352} are even conserved in eukaryotic RNase P RNAs (Tranguch and Engelke, 1993). Assuming that highly conserved bases are involved in forming the ribozyme core, most of the identified phosphorothioate positions will reside in this region of the RNA. Although the base identities at positions 72, 353 and 354 are less strictly conserved, these nucleotides are part of the so-called 'cage' region (encompassing helices P1, P2 and P3 and the long-range base-pairing interaction P4; Figure 5), the most conserved structural feature of all RNase P and RNase MRP RNA subunits (Forster and Altman, 1990b). The fact that >50% of the sites, which interfere with tRNA binding, reside within helix P4 supports its involvement in the most intricate part of the core structure and suggests a key role for enzyme function.

A_{317} lies within a hairpin loop (P18, Figure 5), whose exact orientation in the three-dimensional structure has been uncertain (Harris *et al.*, 1994). Deletion variants of RNase P RNAs from *E.coli* (Haas *et al.*, 1994) and *T.thermophilus* (Schlegl *et al.*, 1994) lacking the hairpin structure comprising A_{317} had increased K_ms for the substrate at lower salt concentrations. The terminal loop of P18 belongs to the family of 5'-GNRA tetraloops, which are frequently found in natural RNAs (Woese *et al.*, 1990). Co-variation analyses of self-splicing group I introns and biochemical evidence suggested that nucleotides of the tetraloop may interact with specific base pairs in the minor groove of helices (Michel and Westhof, 1990; Jaeger *et al.*, 1994; Murphy and Cech, 1994). Furthermore, in crystals of a hammerhead RNA–DNA ribozyme–inhibitor complex, the tetraloop 5'-GAAA (connecting the strands of stem II) of one molecule interacts with the minor groove of stem II of a second molecule (Pley *et al.*, 1994). The *pro*-Rp oxygen of the A on the 3'-side of this tetraloop is hydrogen-bonded to the exocyclic amino group of the G on its 5'-side. Interestingly, the phosphorothioate interference found at A_{317} of the RNase P RNA P18 tetraloop in this study is located at an analogous position (Figure 5), and there is phylogenetic evidence for hydrogen-bonding of G_{316} with the base pair A_{94}:U_{104} in helix P8 (James W.Brown, personal communication). Thus, the *pro*-Rp oxygen 5' of A_{317} may interact with the 2-amino group of G_{314}, thereby stabilizing the loop conformation and, by indirect means, supporting the long-range interaction with a duplex minor groove. Based on increasing evidence for the important role of tetraloops in RNA tertiary structure, it is startling that we observed interference only in one of the four GNRA loops of *E.coli* RNase P RNA.

No sites of interference were detected in the region of nucleotides 133–247, which corresponds to almost one-third of the catalytic RNA (Figure 5). In a deletion study of the RNase P RNA from *T.thermophilus*, removal of the nucleotides roughly corresponding to positions 138–228 of *E.coli* RNase P RNA had a relatively moderate effect on catalytic activity (Schlegl *et al.*, 1994). Likewise, simultaneous deletion of P12, P13 and P14 (Figure 5) in *E.coli* RNase P RNA had only a marginal effect on catalytic activity when assayed at 3 M ammonium acetate (Darr *et al.*, 1992). These observations suggest that the region comprising elements P12, P13 and P14 is of lower importance for RNase P RNA function *in vitro* in the absence of the protein subunit.

It is further noteworthy that we did not observe sites of phosphorothioate interference in the region of nucleotides 253–296 (Figure 5). Chemical footprinting studies, using base-specific reagents such as dimethylsulfate and kethoxal, revealed protection of *E.coli* RNase P RNA from modification at nucleotides A_{254}, A_{255}, G_{259}, G_{292} and G_{293} in the presence of tRNA. However, protection at these sites was abolished for a tRNA lacking the CCA 3' end, suggesting that these contacts are specific for the tRNA 3' end (LaGrandeur *et al.*, 1994). This is in line with the recent finding that tRNA nucleotides C_{74} and C_{75} can base-pair with G_{293} and G_{292} of *E.coli* RNase P RNA (Kirsebom and Svärd, 1994). Evidence for a specific interaction of the CCA end with nucleotides of the internal loop region enclosed by helices P15 and P16 (Figure 5) comes also from cross-linking studies (Oh and Pace, 1994). In addition, four prominent sites of metal ion-induced hydrolysis could be assigned to the region of nucleotides 253–296 in *E.coli* RNase P RNA (Kazakov and Altman, 1991; Zito *et al.*, 1993; Ciesiolka *et al.*, 1994), and the cleavage pattern of Pb^{2+}-induced hydrolysis at three of these sites changed upon tRNA binding (Ciesiolka *et al.*, 1994). Furthermore, it has been proposed that the internal loop of nucleotides 254–259 and 291–295, which harbours two of the metal ion-induced sites of hydrolysis, maintains the structure or may be part of the catalytic centre (Kazakov and Altman, 1991). Our failure to observe Rp-phosphorothioate interferences in this region of the catalytic RNA suggests that metal ion coordination relevant to tRNA binding and contacts to the mature tRNA domain in this region of RNase P RNA are likely to depend on other functional groups than *pro*-Rp oxygens, such as the base moieties of G_{293} and G_{292} (Kirsebom and Svärd, 1994).

Relation to previous biochemical data

Photocross-linking with azidophenacyl-conjugated tRNAs has been used to characterize the spatial arrangements within the tRNA–RNase P RNA complex. The data indicate that the tRNA 5'-end is located within a range of 10 Å to nucleotides A_{248}, A_{249} and A_{330} (Burgin and Pace, 1990). Likewise, the tRNA 3'-end was inferred to be close to nucleotides 331–333 (Oh and Pace, 1994), and nucleotides 64–67 of *E.coli* RNase P RNA were among those that cross-linked with azidophenacyl groups conjugated to nucleotides 64 and 69 of the tRNA (Nolan *et al.*,

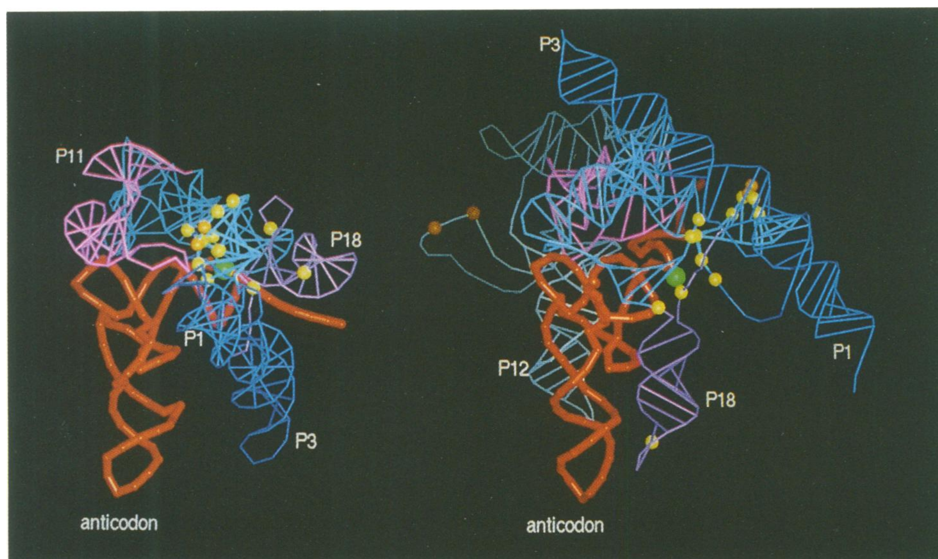

Fig. 6. Tertiary structure models of the pre-tRNA–*E.coli* RNase P RNA complex. Rp-phosphorothioate modifications that interfered with the binding of $tRNA^{Gly}_{(intron)}$ (yellow and orange spheres) are shown in the context of the two models. Orange spheres indicate positions that could be rescued by the addition of manganese. Pre-tRNAs are shown in red and the scissile phosphodiester bond is marked in green. The model shown on the left was kindly provided by M.Harris, N.R.Pace and their colleagues (Harris *et al.*, 1994); the model shown on the right was kindly provided by E.Westhof and S.Altman (Westhof and Altman, 1994). Dark blue, helices P1, P2 and P3 (Figure 5); light blue, helices P4, P5, P6, P15, P16 and P17; purple, P18 and flanking single-stranded nucleotides; pink, helices P7, P8, P9 and P10; blue–grey, nucleotides 123–234, which are missing in the model on the left.

1993; Harris *et al.*, 1994). Thus, it is likely that *pro*-Rp oxygens at A_{67}, A_{248}, A_{249} and A_{330}, for which we observed phosphorothioate interference, are positioned at the tRNA–RNase P RNA interface.

According to the available cross-linking data, the location of positions A_{130} and A_{132} is less certain with respect to the (pre-)tRNA. The absence of photocross-links to a certain position, however, does not necessarily argue for a more distant location, since cross-linking efficiency can depend on the orientation of the photoreactive group and the local chemical environment.

The distance constraints obtained from intramolecular RNase P RNA cross-links and intermolecular cross-links using precursor and mature tRNA derivatives were incorporated into a three-dimensional model of the pre-tRNA–RNase P RNA complex (Harris *et al.*, 1994; Figure 6, left). No distinction between binding of mature versus precursor tRNA was made. In this model, each nucleotide is represented by one pseudoatom. Note that helix P3 is shortened and nucleotides 123–234 are not present in the current form of this model. A second working model was published in parallel by Westhof and Altman (1994; Figure 6, right). Both models are shown in roughly the same orientation based on equal positioning of the tRNA anticodon arms. Positions of phosphorothioate interference obtained for the binding of mature $tRNA^{Gly}_{(intron)}$ to *E.coli* RNase P RNA are shown in the context of the two three-dimensional models for the pre-tRNA–RNase P RNA complex (Figure 6). It is noteworthy that the sites of interference found in the study presented here are clustered in the vicinity of the scissile bond (marked in green) in both models. According to the model by Harris *et al.* (1994), phosphates 5′ of A_{67}, G_{68}, A_{248}, A_{249}, A_{330}, A_{352}, C_{353} and C_{354} would be within a distance of 15 Å to the scissile phosphodiester bond, and those at U_{69}, C_{70}, C_{71}, G_{72} and G_{300} within a distance of 25 Å. In the model by Westhof and Altman, only phosphates 5′ of A_{330}, A_{352}, C_{353} and C_{354} fall into the 15 Å range with respect to the scissile phosphodiester. These four positions reside in two regions, whose deletion from the RNase P RNA of *T.thermophilus* had the most dramatic effects on catalytic activity (Schlegl *et al.*, 1994), suggesting a key role for enzyme function.

Sites of metal ion coordination

Phosphorothioate interference with $tRNA^{Gly}_{(intron)}$ binding was rescued at positions A_{130} and A_{132} as well as at U_{69} and C_{70} when the binding assay was performed at 5 mM Mn^{2+}/95 mM Mg^{2+}. The Mn^{2+} rescue indicates that the *pro*-Rp oxygens at these sites are directly coordinated to Mg^{2+} ions that are required for high-affinity binding of tRNA. These results provide the first information on functional groups of RNase P RNA involved in the complexing of specific Mg^{2+} ions. Thus far, it remains unclear whether these Mg^{2+} ions may also exert a function in active site chemistry.

In analogy to yeast $tRNA^{Phe}$ (Holbrook *et al.*, 1977; Jack *et al.*, 1977), the sites of manganese rescue found in our study (A_{130}, A_{132} and U_{69}, C_{70}) may be due to two strong binding sites for $[Mg(H_2O)_4]^{2+}$ coordinated directly to pairs of *pro*-Rp oxygens at A_{130}, A_{132} and U_{69}, C_{70}, respectively. At least two additional Mg^{2+} ions were shown to be bound upon tRNA–RNase P RNA complex formation (Hardt *et al.*, 1993b). It is tempting to speculate that *pro*-Rp oxygens at A_{130}, A_{132}, U_{69} and C_{70} are involved in the coordination of these metal ions. Additional sites of metal ion coordination in the RNase P RNA core (besides U_{69} and C_{70}) involving non-bridging phosphate oxygens may have remained undetected in this study, for the following reasons: (i) *pro*-Sp oxygens were not analysed, (ii) magnesium binding sites may also include hydrogen-bonded magnesium hydrates of the

$[Mg(H_2O)_6]^{2+}$ type (as reported for yeast $tRNA^{Phe}$; Holbrook *et al.*, 1977), which lack a direct coordination to phosphate oxygens, and thus are likely to be insensitive to manganese rescue and (iii) at least some *pro*-Rp oxygens involved in direct or hydrate-mediated metal ion coordination may have escaped detection due to the high magnesium concentrations used. Higher concentrations of Mg^{2+} were found to compensate for the ion's reduced affinity for sulfur at a few positions of weak interference in a study of the *Tetrahymena* group I intron (Christian and Yarus, 1993).

Clustering of Rp-phosphorothioate interferences in catalytic RNAs

In *E.coli* RNase P RNA, sequential sites of phosphorothioate interference occurred in the region of helix P4 (Figure 5). A similar clustering of functionally important *pro*-Rp oxygens was observed for the *Tetrahymena* ribozyme in the region of helix P7 (Christian and Yarus, 1992). Interestingly, helix P7 of this ribozyme, like helix P4 of RNase P RNA (Figure 5), includes a bulged nucleotide, and formation of helix P7 (and helix P3) is rate-limiting in the tertiary folding of the *Tetrahymena* ribozyme (Zarrinkar and Williamson, 1994). For the hepatitis delta virus (HDV) ribozyme, a clustering of Rp-phosphorothioates that interfered with the self-cleavage reaction was observed in stem I, whose spatial relationship to the functionally important stem/loop III seems to be critical for activity (Jeoung *et al.*, 1994; Tanner *et al.*, 1994). In conclusion, the clustering of Rp-phosphorothioate interferences in some helices of catalytic RNAs seems to indicate the critical role of these structural elements for tertiary folding.

It should be noted that phosphorothioate interferences do not necessarily imply that *pro*-Rp oxygens at these locations are directly involved in H-bonding. For example, phosphorothioate substitution can destabilize DNA helices, and this effect was shown to be more pronounced for Rp- than Sp-modifications (Stec and Wilk, 1994). This is likely to be related to the fact that Rp-substitutions point to the helix interior. At some positions, the larger sphere of sulfur or the non-identical charge localization in phosphorothioates versus phosphates (Frey and Sammons, 1985) might force the backbone into different conformations, which may result in misfolding or instability of RNase P RNA.

Relation to sites of Pb^{2+}-induced hydrolysis

Several prominent sites of metal ion-induced hydrolysis, attributable to specific metal ion binding centres, were identified in *E.coli* RNase P RNA (Kazakov and Altman, 1991; Zito *et al.*, 1993; Ciesiolka *et al.*, 1994). However, the sites of metal ion coordination and induced hydrolysis may be remote from each other in the primary sequence, as known for yeast $tRNA^{Phe}$ (Rubin and Sundaralingam, 1983; Brown *et al.*, 1985). Since little is known about the structural relation of the 'upper loop' region (nucleotides 123–234, Figure 5) of RNase P RNA and the remainder of the RNA, the non-bridging *pro*-Rp oxygens of nucleotides A_{130} and A_{132} may coordinate any of the Pb^{2+} ions that cause site-specific cleavage in *E.coli* RNase P RNA (Figure 5, highlighted nucleotides). So far, both RNase P RNA models (Figure 6) give no final clues regarding the relation of Pb^{2+}-hydrolysis sites and metal ion coordination to *pro*-Rp oxygens at A_{130}/A_{132} and U_{69}/C_{70}. Nevertheless, the importance of metal ion coordination to *pro*-Rp oxygens at A_{130}/A_{132} for tRNA binding in concert with the observation that Pb^{2+}-induced hydrolysis at A_{234} is reduced due to complex formation with tRNA (Ciesiolka *et al.*, 1994) indicates a functional linkage of the 'upper loop' region to the rest of the catalytic RNA.

Rp-phosphorothioate modifications of tRNA had little effect on binding to RNase P RNAs

Complete Rp-phosphorothioate modification of mature *S.cerevisiae* $tRNA^{Phe}$ or *T.thermophilus* $tRNA^{Gly}$ had only moderate effects on binding affinity to RNase P RNAs from *E.coli*, *B.subtilis* and *C.vinosum* (data not shown) compared with the drastic decrease in catalytic efficiency and tRNA binding affinity that we have observed with fully modified *E.coli* RNase P RNAs (Figure 1). This is in line with studies of the pre-tRNA cleavage reaction which revealed that Rp-phosphorothioates had only strong effects on pre-tRNA processing and/or cleavage site selection when present at the scissile phosphodiester bond (Kahle *et al.*, 1993).

The main recognition elements for *E.coli* RNase P RNA are located in the quasi-continuous helix formed by acceptor stem and T-stem of tRNAs (McClain *et al.*, 1987; Forster and Altman, 1990a; Kahle *et al.*, 1990; Thurlow *et al.*, 1991). Since *pro*-Sp oxygens and ribose 2'-OH groups are directed towards the helix surface, they are more likely candidates for hydrogen bonding to the enzymatic RNA than *pro*-Rp oxygens.

In conclusion, the binding interference approach, which allowed separation of binding and non-binding RNase P RNA fractions, has proven useful in identifying *pro*-Rp oxygens whose replacement with sulfur impairs specific complex formation with tRNA molecules. *Pro*-Rp oxygens of tRNAs were found to be of lower importance for complex formation. The binding interference assay provides a powerful tool to screen a variety of other chemical modifications in order to improve our understanding of the molecular details of tRNA recognition by RNase P RNA. In concert with cleavage interference studies, one should be able to define functional groups, including sites of metal ion coordination, that are relevant to (pre-)tRNA binding or catalysis or both.

Materials and methods

DNA templates

Templates for T7 transcription of *E.coli* RNase P RNA, mature $tRNA^{Gly}$ endowed with the 60 nucleotide intron of *S.cerevisiae* $tRNA^{Ile}$ ($tRNA^{Gly}{}_{(intron)}$; see Hardt *et al.*, 1993b), mature $tRNA^{Phe}$ from *S.cerevisiae* and mature $tRNA^{Gly}$ and pre-$tRNA^{Gly}$ from *T.thermophilus* were prepared by PCR (Hardt *et al.*, 1993b). T7 transcription vectors encoding the RNase P RNAs from *B.subtilis* and *C.vinosum* (kindly provided by N.R.Pace) were prepared by standard methods and linearized by *Dra*I and *Cla*I digestion, respectively.

Preparation of RNAs

Unmodified RNase P RNA from *E.coli*, pre-$tRNA^{Gly}$, mature $tRNA^{Gly}$ and mature $tRNA^{Gly}{}_{(intron)}$ were synthesized by run-off transcription with T7 RNA polymerase as described, including 9 mM 5'-GMP for the synthesis of mature tRNAs (Schlegl *et al.*, 1992; Hardt *et al.*, 1993b). Transcription of RNase P RNAs from *B.subtilis* and *C.vinosum* was performed with 4 mM each of ATP, CTP and GTP and 1 mM UTP. After DNase I digestion, transcription assays were extracted with

phenol:chloroform (1:1), and tRNAs were purified on 8% polyacrylamide–8 M urea gels, localized by UV shadowing, excised from the gel, eluted overnight at 4°C in 200 mM Tris–HCl, pH 7.1, 1 mM EDTA and recovered by ethanol precipitation in the presence of 75 mM NaOAc, pH 6.7. RNase P RNAs were purified by Sephadex-G50 gel filtration and recovered by ethanol precipitation. RNAs were dissolved in water and concentrations were determined by absorbance at 260 nm (1 A_{260} unit = 37 μg).

Phosphorothioate-modified RNAs were prepared by T7 transcription as described (Kahle *et al.*, 1993). NTP concentrations for the synthesis of fully modified RNAs were 1 mM each, except that one NTP species was replaced with its Sp-NTPαS analogue (1 mM); in addition, assays contained either 4 mM GMP (to synthesize mature tRNAs with 5′-monophosphates) or 4 mM ApG (to facilitate 5′-end labelling of RNase P RNA) as initiators of transcription. For the synthesis of radioactively labelled, fully modified tRNAs, [α-^{32}P]CTP (for 100% modification 5′ of A, G and U) or [α-^{32}P]ATP (for 100% modification 5′ of C) was included. Partially modified RNase P RNAs were prepared by T7 transcription in the presence of ATP, CTP, GTP and UTP (each 1 mM), 4 mM ApG and indicated concentrations of the respective Sp-NTPαS (given in % of the concentration of the regular NTP). After DNase I digestion, one-third volume of loading buffer [67% formamide, 30% buffer C (buffer C = 90 mM boric acid, 90 mM Tris base, 2 mM EDTA, pH 8.3), 2.7 M urea, 100 mM EDTA] was added, and assays were loaded directly on a denaturing polyacrylamide gel (5% for RNase P RNAs; 9% for tRNAs) containing 8 M urea. Gel elution and ethanol precipitation were performed as described above.

3′- and 5′-end labelling

3′-ends of *E.coli* RNase P RNA were labelled with 5′-[^{32}P]pCp using T4 RNA ligase (England and Uhlenbeck, 1978). 5′-end labelling was performed with [γ-^{32}P]ATP and T4 RNA kinase (Maniatis *et al.*, 1982) using RNAs with free 5′-OH groups (see preparation of RNAs). The labelled material was purified on denaturing polyacrylamide gels, bands were visualized by autoradiography, excised and eluted as described above. Ethanol precipitation was performed in the presence of 3 μg/ml 50S ribosomal RNA from *E.coli*. Samples were dissolved in 10 μl water and stored at –20°C.

Processing assays

Pre-tRNA^{Gly} internally labelled with ^{32}P was used as the substrate. Processing assays with *E.coli* RNase P RNA were performed at 37°C in buffer A [100 mM $Mg(OAc)_2$, 100 mM NH_4OAc and 1 mM EDTA, 50 mM TrisOAc, pH 6.6, at 37°C] or buffer B [5 mM $Mn(OAc)_2$, which was always freshly dissolved to avoid oxidation, 95 mM $Mg(OAc)_2$, 100 mM NH_4OAc and 1 mM EDTA, 50 mM TrisOAc, pH 6.6, at 37°C]. Enzymes and substrates were pre-incubated separately for 60 min in buffer A or buffer B and reactions were started by combining pre-warmed enzyme and substrate solutions. Aliquots were withdrawn at different time points, and reactions were stopped by adding 1.3 volumes of loading buffer and shock-freezing in liquid nitrogen. Maturation of pre-tRNA (15 000 Cerenkov c.p.m. per lane) was analysed on denaturing 10% polyacrylamide gels. Bands corresponding to pre-tRNA and mature tRNA were excised from the gels and quantified by Cerenkov counting.

Gel retardation

Basically, assays were performed as described (Hardt *et al.*, 1993a,b). Excess amounts of *E.coli*, *B.subtilis* or *C.vinosum* RNase P RNAs were preincubated at 37°C for 60 min in 9 μl 1.1× buffer A or B (see above) including 5.6% glycerol. One microlitre of trace amounts of ^{32}P-labelled tRNA (<5 nM) was added, and assays were incubated for 30 min at 37°C. Samples were loaded on 7.5% polyacrylamide gels equilibrated in buffer A or buffer B and pre-warmed to 37°C. Separation of bound and unbound tRNA was accomplished within 3 h. Gels were dried on Whatman 3MM paper before autoradiography; bands were excised and the radioactivity was quantified by Cerenkov counting; radioactivity above the band corresponding to free tRNA was attributed to the fraction of complexed tRNA to also account for complexes which dissociated during electrophoresis. To assay tRNA$^{Gly}_{(intron)}$ binding to partially phosphorothioate-modified *E.coli* RNase P RNAs, 1.2 μM ^{32}P-labelled RNase P RNA and 1.9 μM mature tRNA$^{Gly}_{(intron)}$ (if not stated otherwise) were incubated for 60 min in buffer A or B containing 5% glycerol and were run on a native 7.5% gel for 12 h, as described above. The running buffer was exchanged several times to ensure constant buffer A or buffer B conditions during electrophoresis.

Iodine hydrolysis

End-labelled RNAs were dissolved in 9 μl 11 mM HEPES, pH 7.4. Iodine cleavage was started by addition of 1 μl iodine solution (1 mg/ml in ethanol). After 10 min incubation at 37°C, the reaction was stopped by ethanol precipitation. The samples were dissolved in 8 μl loading buffer and analysed on 5, 6, 8 or 10% polyacrylamide gels containing 8 M urea. Cleavage products were visualized by autoradiography.

Acknowledgements

We thank Guido Krupp for his expert advice concerning the iodine-induced cleavage of phosphorothioates, Thomas Specht and Michael Assmann for their expert help with the computer graphic work, and Rolf Bald for the synthesis of ApG. We are indebted to James W.Brown for providing unpublished data, Michael Harris and Norman R.Pace, and Eric Westhof and Sidney Altman for providing the coordinates of their RNase P models. We also thank N.R.Pace and E.Westhof for helpful discussions. The work has been supported by the Deutsche Forschungsgemeinschaft (SFB 344/D6/C2 and Ha 1672/4–1). W.-D.H. is a stipend of the Boehringer Ingelheim Fonds.

References

Altman,S., Kirsebom,L. and Talbot,S. (1993) *FASEB J.*, **7**, 7–14.
Brown,R.S., Dewan,J.C. and Klug,A. (1985) *Biochemistry*, **24**, 4785–4801.
Burgin,A.B. and Pace,N.R. (1990) *EMBO J.*, **9**, 4111–4118.
Ciesiolka,J., Hardt,W.-D., Schlegl,J., Erdmann,V.A. and Hartmann,R.K. (1994) *Eur. J. Biochem.*, **219**, 49–56.
Christian,E.L. and Yarus,M. (1992) *J. Mol. Biol.*, **228**, 743–758.
Christian,E.L. and Yarus,M. (1993) *Biochemistry*, **32**, 4475–4480.
Darr,S.C., Zito,K., Smith,D. and Pace,N.R. (1992) *Biochemistry* **31**, 328–333.
Drainas,D., Zimmerly,S., Willis,I. and Söll,D (1989) *FEBS Lett.*, **251**, 84–88.
England,T.E. and Uhlenbeck,O.C. (1978) *Nature*, **275**, 560–561.
Forster,A.C. and Altman,S. (1990a) *Science*, **249**, 783–785.
Forster,A.C. and Altman,S. (1990b) *Cell*, **62**, 407–409.
Frank,D.N., Harris,M.E. and Pace,N.R. (1994) *Biochemistry*, **33**, 10800–10808.
Frey,P.A. and Sammons,R.D. (1985) *Science*, **228**, 541–545.
Gaur,R.K. and Krupp,G. (1993) *Nucleic Acids Res.*, **21**, 21–26.
Green,C.J. and Vold,B.S. (1988) *J. Biol. Chem.*, **263**, 652–657.
Griffiths,A.D., Potter,B.V.L. and Eperon,I.C. (1987) *Nucleic Acids Res.*, **15**, 4145–4162.
Guerrier-Takada,C., Gardiner,K., Marsh,T., Pace,N. and Altman,S. (1983) *Cell*, **35**, 849–857.
Guerrier-Takada,C., McClain,W.H. and Altman,S. (1984) *Cell*, **38**, 219–224.
Guerrier-Takada,C., Lumelsky,N. and Altman,S. (1989) *Science*, **246**, 1578–1584.
Haas,E.S., Brown,J.W., Pitulle,C. and Pace,N.R. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 2527–2531.
Hardt,W.-D., Schlegl,J., Erdmann,V.A. and Hartmann,R.K. (1993a) *Biochemistry*, **32**, 13046–13053.
Hardt,W.-D., Schlegl,J., Erdmann,V.A. and Hartmann,R.K. (1993b) *Nucleic Acids Res.*, **21**, 3521–3527.
Hardt,W.-D., Schlegl,J., Erdmann,V.A. and Hartmann,R.K. (1995) *J. Mol. Biol.*, **247**, 161–172.
Harris,M.E., Nolan,J.M., Malhotra,A., Brown,J.W., Harvey,S.C. and Pace,N.R. (1994) *EMBO J.*, **13**, 3953–3963.
Holbrook,S.R., Sussman,J.L., Warrant,R.W., Church,G.M. and Kim,S.-H. (1977) *Nucleic Acids Res.*, **4**, 2811–2820.
Jack,A., Ladner,J.E., Rhodes,D., Brown,R.S. and Klug,A. (1977) *J. Mol. Biol.*, **111**, 315–328.
Jaffe,E.K. and Cohn,M. (1979) *J. Biol. Chem.*, **254**, 10839–10845.
Jaeger,L., Michel,F. and Westhof,E. (1994) *J. Mol. Biol.*, **236**, 1271–1276.
Jeoung,Y.-H., Kumar,P.K.R., Suh,Y.-A., Taira,K. and Nishikawa,S. (1994) *Nucleic Acids Res.*, **22**, 3722–3727.
Kahle,D., Wehmeyer,U. and Krupp,G. (1990) *EMBO J.*, **9**, 1929–1937.
Kahle,D., Küst,B. and Krupp,G. (1993) *Biochimie*, **75**, 955–962.
Kazakov,S. and Altman,S. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9193–9197.
Kikuchi,Y., Sasaki-Tozawa,N. and Suzuki,K. (1993) *Nucleic Acids Res.*, **21**, 4685–4689.
Kirsebom,L.A. and Svärd,S.G. (1993) *J. Mol. Biol.*, **231**, 594–604.

Kirsebom,L.A. and Svärd,S.G. (1994) *EMBO J.*, **13**, 4870–4876.
Knap,A.K., Wesolowski,D. and Altman,S. (1990) *Biochimie*, **72**, 779–790.
LaGrandeur,T.E., Hüttenhofer,A., Noller,H.F. and Pace,N.R. (1994) *EMBO J.*, **13**, 3945–3952.
Leontis,N., DaLio,A., Strobel,M. and Engelke,D. (1988) *Nucleic Acids Res.*, **16**, 2537–2552.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mattsson,J.G., Svärd,S.G. and Kirsebom,L.A. (1994) *J. Mol. Biol.*, **241**, 1–6.
McClain,W.H., Guerrier-Takada,C. and Altman,S. (1987) *Science*, **238**, 527–530.
Michel,F. and Westhof,E. (1990) *J. Mol. Biol.*, **216**, 585–610.
Murphy,F.L. and Cech,T.R. (1994) *J. Mol. Biol.*, **236**, 49–63.
Nolan,J.M., Burke,D.H. and Pace,N.R. (1993) *Science*, **261**, 762–765.
Nichols,M., Söll,D. and Willis,I. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 1379–1383.
Oh,B.-K. and Pace,N.R. (1994) *Nucleic Acids Res.*, **22**, 4087–4094.
Pecoraro,V.L., Hermes,J.D. and Cleland,W.W. (1984) *Biochemistry*, **23**, 5262–5271.
Pley,H.W., Flaherty,K.M. and McKay,D.B. (1994) *Nature*, **372**, 111–113.
Reich,C., Olsen,G.J., Pace,B. and Pace,N.R. (1988) *Science*, **239**, 178–181.
Rubin,J.R. and Sundaralingam,M. (1983) *J. Biomolec. Struct. Dynam.*, **1**, 639–646.
Sampson,J.R. and Uhlenbeck,O.C. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 1033–1037.
Schlegl,J., Fürste,J.P., Bald,R., Erdmann,V.A. and Hartmann,R.K. (1992) *Nucleic Acids Res.*, **20**, 5963–5970.
Schlegl,J., Hardt,W.-D., Erdmann,V.A. and Hartmann,R.K. (1994) *EMBO J.*, **13**, 4863–4869.
Schmitt,M.E., Bennett,J.L., Dairaghi, D.J. and Clayton,D.A. (1993) *FASEB J.*, **7**, 208–213.
Smith,D., Burgin,A.B., Haas,E.S. and Pace,N.R. (1992) *J. Biol. Chem.*, **267**, 2429–2436.
Stec,W.J. and Wilk,A. (1994) *Angew. Chem. Int. Ed.*, **106**, 709–712.
Svärd,S.G. and Kirsebom,L.A. (1992) *J. Mol. Biol.*, **227**, 1019–1031.
Svärd,S.G. and Kirsebom,L.A. (1993) *Nucleic Acids Res.*, **21**, 427–434.
Tallsjö,A. and Kirsebom,L.A. (1993) *Nucleic Acids Res.*, **21**, 51–57.
Tanner,N.K., Schaff,S., Thill,G., Petit-Koskas,E., Crain-Denoyelle,A.-M. and Westhof,E. (1994) *Curr. Biol.*, **4**, 488–498.
Thurlow,D.L., Shilowski,D. and Marsh,T.L. (1991) *Nucleic Acids Res.*, **19**, 885–891.
Tranguch,A.J. and Engelke,D.R. (1993) *J. Biol. Chem.*, **268**, 14045–14053.
Wang,M.J., Davis,N.W. and Gegenheimer,P. (1988) *EMBO J.*, **7**, 1567–1574.
Westhof,E. and Altman,S. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 5133–5137.
Woese,C.R., Winker,S. and Gutell,R.R. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 8467–8471.
Yuan,Y., Hwang,E.S. and Altman,S. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8006–8010.
Yuan,Y. and Altman,S. (1995) *EMBO J.*, **14**, 159–168.
Zarrinkar,P.P. and Williamson,J.R. (1994) *Science*, **265**, 918–923.
Zito,K., Hüttenhofer,A. and Pace,N.R. (1993) *Nucleic Acids Res.*, **21**, 5916–5920.

Received on January 30, 1995; revised on March 27, 1995