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Mapping the active site of ribonuclease P RNA using a substrate containing a photoaffinity agent

Alex B.Burgin and Norman R.Pace

Department of Biology and Institute for Molecular and Cellular Biology, Indiana University, Bloomington, IN 47405, USA

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Ribonuclease P RNA is the catalytic moiety of the ribonucleoprotein enzyme that removes precursor sequences from 5'-ends of pre-tRNAs. A photoaffinity cross-linking agent was coupled to the substrate phosphate on which RNase P acts and used to map nucleotides in the vicinity of the catalytic site of this ribozyme. Mature $tRNA^{Phe}$ containing a 5'-thiophosphate was synthesized by transcription *in vitro* using phage T7 RNA polymerase in the presence of guanosine 5'-phosphorothioate. The photoagent (azidophenacyl) was coupled uniquely to the 5'-thiophosphate of the tRNA, the site of action by RNase P. The photoagent-containing tRNA binds to RNase P RNA and is cross-linked by UV irradiation to it at high efficiency (10–30%). Cross-linked conjugates are enzymatically inactive, consistent with the occupancy of the active site of the RNase P RNA by the tRNA. Reversal of the cross-link by phenylmercuric acetate restores activity. The sites of cross-linking in RNase P RNA were determined by primer extension. In order to identify generalities and detect idiosyncrasies, analyses were carried out using RNase P RNAs from three phylogenetically diverse organisms: *Bacillus subtilis, Chromatium vinosum* and *Escherichia coli*. In the context of a phylogenetic structure model, two regions of cross-linking are observed in all three RNAs. Two of the RNAs cross-link to a lesser extent at a third structural region and one of the RNAs is cross-linked to a small extent to a fourth region. All the sites of cross-linking between the substrate phosphate in tRNA and the RNase P RNAs are in the conserved core of the structure model, consistent with the importance of the cross-linked residues to the action of this RNA enzyme.

Key words: photoaffinity cross-linking/ribonuclease P/RNase P RNA/ribozyme/tRNA processing

Introduction

Ribonuclease P (RNase P) is a universally required endoribonuclease that cleaves tRNA precursors (pre-tRNAs) to produce the mature 5'-ends (Altman, 1989; Pace and Smith, 1990 for recent reviews). In all eubacteria so far examined, RNase P is composed of protein and RNA elements (Stark *et al.*, 1978; Gardiner and Pace, 1980). However, at high salt concentrations *in vitro* RNase P RNA alone is an efficient and accurate catalyst (Guerrier-Takada *et al.*, 1983). The high salt (*in vitro*) and the RNase P protein (*in vivo*) are believed to screen electrostatic repulsion between the RNA enzyme and the pre-tRNA substrate, facilitating binding and hence cleavage (Gardiner *et al.*, 1985; Reich *et al.*, 1988).

A secondary structure model of the eubacterial RNase P RNA has been developed by using phylogenetic comparisons (James *et al.*, 1988; Pace *et al.*, 1989). Although there is substantial sequence variation among the examined eubacterial RNase P RNAs, a common core of homologous sequence and secondary structure is identifiable. It is clear that this conserved core structure contains the catalytic elements of the RNA enzyme: a simplified RNase P RNA (*Min* 1 RNA) containing only the phylogenetically conserved features is an accurate catalyst of the RNase P reaction, with nearly the same catalytic efficiency (k_{cat}/K_M) as the native RNA (Waugh *et al.*, 1989). In addition, deletions of conserved elements in the RNase P RNA result in greatly reduced activity (Guerrier-Takada and Altman, 1986; Waugh, 1989). These latter results are difficult to interpret, however, since it is not clear whether the deletions remove catalytic residues or simply disrupt the structure of the RNA enzyme and thereby prevent catalysis.

In order to map more precisely the active site of RNase P RNA, we have used photoaffinity cross-linking to identify residues in the RNase P RNA that are adjacent to the substrate phosphodiester bond in pre-tRNA. As outlined in Figure 1, an azidophenacyl group was attached to the 5'-phosphate of mature tRNA, the phosphate acted upon by RNase P. The azidophenacyl group is a photoactivatable cross-linking agent (Hixson and Hixson, 1975). Upon UV irradiation the azido moiety is converted to a nitrene, which then inserts into nearby bonds. Mature tRNA is a competitive inhibitor of RNase P and it binds to the ribozyme as efficiently as does the pre-tRNA ($K_i \approx K_M$) (Reich *et al.*, 1988; Reich, 1988). The binding of photoagent-containing tRNA is therefore expected to position the photoagent at or near the active site of RNase P RNA. The phenacyl nitrene group is fairly small (~ 9 Å in length), so in principle it is a cross-linking probe for the identification of RNase P RNA residues that are in the immediate proximity of the substrate bond during the reaction.

Results

Synthesis of azidophenacyl tRNA

In order to couple a cross-linking agent specifically at the 5'-phosphate of mature tRNA, we first synthesized mature $tRNA^{Phe}$ in which the 5'-terminus is a thiophosphate. The thio-containing tRNA then was coupled with azidophenacyl bromide by nucleophilic displacement of bromine. Specific incorporation of the thiophosphate was accomplished by *in vitro* transcription of a $tRNA^{Phe}$ gene in the presence of guanosine 5'-monophosphorothioate (GMPS). Nucleoside monophosphates can prime RNA synthesis (be incorporated as the first nucleotide) by bacteriophage T7 RNA polymerase *in vitro*, but triphosphates are required for chain elongation (Sampson and Uhlenbeck, 1988). GMPS is an efficient

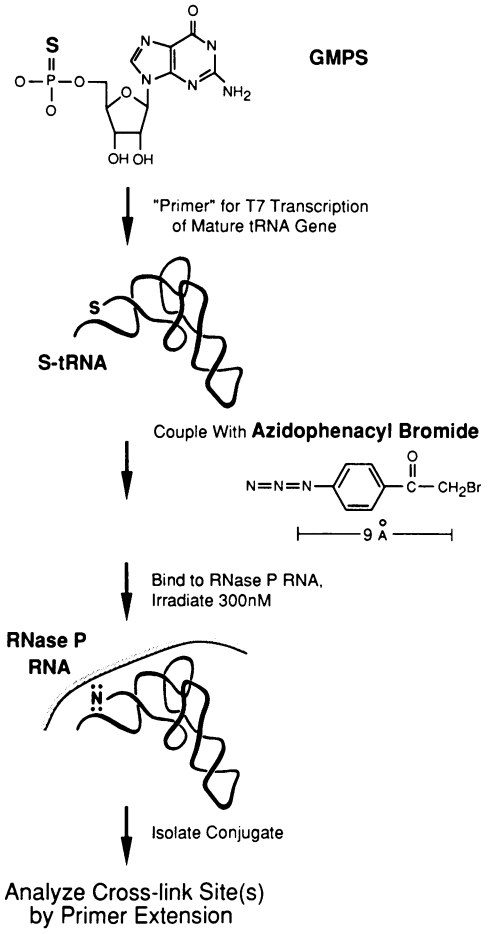

Fig. 1. Experimental outline. Guanosine 5′-phosphorothioate (GMPS) was used as a primer (initiating nucleotide) for *in vitro* transcription of mature $tRNA^{Phe}$ using bacteriophage T7 RNA polymerase. The mature tRNA containing a 5′-terminal thiophosphate (the phosphate acted upon by RNase P) was coupled through the thiophosphate to a photoactivatable cross-linking agent, azidophenacyl bromide (Hixson and Hixson, 1975; Hanna and Meares, 1983). The photoagent-containing tRNA was incubated with excess RNase P RNA and irradiated with 300 nM UV light, converting the azido group to a nitrene. The nitrene is extremely reactive, inducing cross-linking of the tRNA to the RNase P RNA. The cross-linked RNase P RNA–tRNA conjugates were then purified and the sites of cross-linking in the RNase P RNA identified by primer extension analysis.

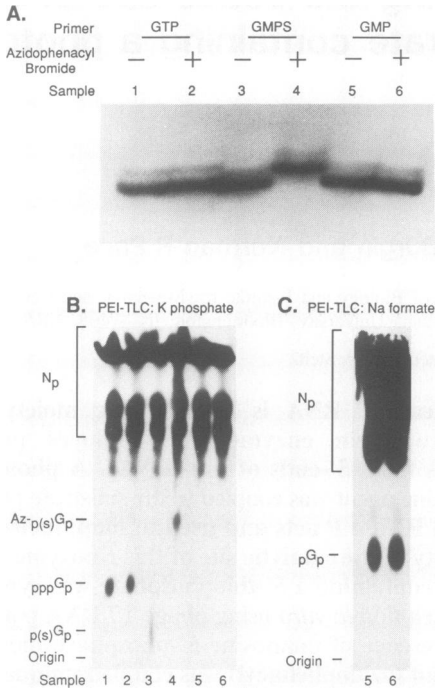

Fig. 2. Azidophenacyl bromide couples with thio-containing rRNA. (**A**) Uniformly labeled ([α-^{32}P]CTP) *E.coli* $tRNA^{Phe}$ was synthesized *in vitro* using T7 RNA polymerase (Materials and methods). Transcriptions were carried out in the presence of ATP, CTP and UTP (1 mM each); and 5′-GMPS ([GMPS] = 4 mM, [GTP] = 0.2 mM), 5′-GMP ([GMP] = 4 mM, [GTP] = 0.2 mM) or with GTP (1 mM) present. Full-length transcripts were gel purified and then incubated in the presence (+) or absence (−) of azidophenacyl bromide. The excess azidophenacyl bromide was removed and the RNAs were recovered by ethanol precipitation. An aliquot of each reaction product (samples 1–6), was resolved on a 12% polyacrylamide gel containing 8 M urea.

An aliquot of each reaction product was also completely hydrolyzed by alkali: following ethanol precipitation, the RNAs were dried *in vacuo*, resuspended in 0.04 N NaOH, incubated at 37°C for 12 h, and neturalized with HCl. The hydrolysates were then spotted onto polyethyleneimine thin layer plates (Brinkman) and the hydrolysis products resolved by ascending chromatography in 0.75 M KH_2PO_4, pH 3.5 (**B**) or 2 M sodium formate (**C**).

primer of RNA synthesis by T7 RNA polymerase and competes approximately stoichiometrically with GTP for RNA chain initiation (data not shown). As a template for RNA synthesis, we used a T7 transcription vector that contains a $tRNA^{Phe}$ gene positioned at the promoter so that the 5′-end of the mature tRNA is the starting site for transcription (Sampson and Uhlenbeck, 1988; Sampson *et al.*, 1989). The 5′-terminal base of the transcript is guanine. The transcription vector also contains a restriction site at the 3′-end of the tRNA gene so that, following cleavage of the DNA, the *in vitro* 'run-off' transcript possesses the mature 3′-end of the tRNA (CCA).

We verified that azidophenacyl bromide can react only with the GMPS-containing RNA, not with RNA that lacks the sulfur. *In vitro* transcriptions of the $tRNA^{Phe}$ gene were carried out in the presence of 5′-GMPS, 5′-GMP, or with only the nucleoside triphosphates present. In the reactions primed by mononucleotides, the mononucleotide (GMPS or GMP):GTP ratios were 40:1, so that nearly all of the transcripts are expected to have 5′-terminal phosphates. The full-length transcripts were gel-purified and incubated in the presence or absence of azidophenacyl bromide (Materials and methods). The excess photoaffinity agent was removed and equal amounts of the products were resolved on a 12% polyacrylamide gel. As shown in Figure 2A, only the tRNA primed by GMPS and incubated in the presence of azidophenacyl bromide (sample 4) exhibits decreased electrophoretic mobility in the gel. The observed decrease in mobility is consistent with the addition of a single azidophenacyl group to the GMPS-containing tRNA. This reaction is essentially quantitative: there is no detectable, unsubstituted, thio-containing tRNA.

In order to demonstrate directly the presence of the azidophenacyl group on the 5′-end of the GMPS-containing tRNA, aliquots of the reaction products were completely hydrolyzed by alkali, and the resulting ^{32}P-labeled ribonucleotides were analyzed by TLC. The transcripts were labeled with [α-^{32}P]CTP, the second nucleotide in the transcript. Consequently, alkaline hydrolysis [which releases 3′ (or 2′) nucleoside monophosphates] transfers a

radiolabeled phosphate to the 5′-terminal nucleotide of the tRNA, which is released as the 5′,3′(2′) bisphosphate, pGp. Therefore, depending upon whether the initiating nucleotide for transcription was GTP, GMPS or GMP, the hydrolysis products representing the 5′-terminal nucleotides will be $_{ppp}G_p$ (guanosine 5′-triphosphate, 3′-monophosphate), $_{p(S)}G_p$ or $_pG_p$. The 3′-phosphate is radioactively labeled in each of the hydrolytic products. Figure 2B shows that if the RNA was primed with GMPS (sample 3), no detectable pppGp is present. Instead, p(S)Gp is released, showing that the GMPS replaces GTP as the 5′-nucleotide in the *in vitro* transcripts. Additionally, $_{p(S)}G_p$ has altered mobility on polyethyleneimine plates if the transcript was treated with azidophenacyl bromide prior to hydrolysis (sample 4). This demonstrates that the photoaffinity agent is coupled to that 5′-terminal thiophosphate. The 5′-terminal nucleotides that lack sulfur, pppGp and pGp, behave the same with or without prior exposure to azidophenacyl bromide, indicating that they do not react with the azidophenacyl bromide. Resolution of pGp from other products could not be achieved in sodium phosphate buffer (Figure 2B), therefore samples 5 and 6 were resolved in sodium formate (Figure 2C).

Photoagent-containing tRNA cross-links to RNase P RNA

Because mature tRNA is a competitive inhibitor of the RNase P reaction, it is expected that mature tRNA binds to the same site on RNase P RNA as is occupied by pre-tRNA. The 5′-terminal phosphate of mature tRNA should thus be at or near the active site of the RNA enzyme. Therefore, GMPS-primed, mature $tRNA^{Phe}$ coupled with the azidophenacyl group should be a photoactivatable cross-linking probe for the identification of RNase P RNA residues that are in proximity to the substrate phosphodiester bond.

Uniformly labeled $tRNA^{Phe}$, primed with GMPS, was coupled with azidophenacyl bromide. The photoagent-containing tRNA was then incubated with a 10-fold molar excess of unlabeled RNase P RNA from *B.subtilis, C.vinosum* or *E.coli,* and exposed to UV light as detailed in Materials and methods. (These experiments were performed in parallel with RNase P RNAs from phylogenetically disparate organisms in order to generalize findings.) After irradiation, reaction components were precipitated with ethanol and resolved on a 4% polyacrylamide gel. As seen in Figure 3A, irradiation in the presence of each of the three RNase P RNAs results in high-efficiency (10−30% in multiple experiments) formation of multiple, presumably cross-linked, products that exhibit different mobilities in the denaturing gel. The occurrence of multiple products of approximately the same size is consistent with the formation of tRNA cross-links to different sites in each of the RNase P RNAs. The figure also shows that cross-linking of the labeled tRNA is dependent upon the presence of RNase P RNA, photoaffinity agent and UV light; and that the complementary ('antisense') strand of RNase P RNA does not promote cross-linking. High-efficiency cross-linking also depends upon high ionic strength (> 600 mM monovalent cation) and it is sensitive to the RNase P RNA:tRNA stoichiometry (not shown). In addition, the observed cross-linking is inhibited stoichiometrically by the inclusion in the reactions of unlabeled mature or precursor $tRNA^{Phe}$ (data not shown).

In some of the gel lanes in Figure 3A there is a labeled band, denoted tRNA′, that migrates more rapidly than tRNA. The presence of this band is dependent upon the

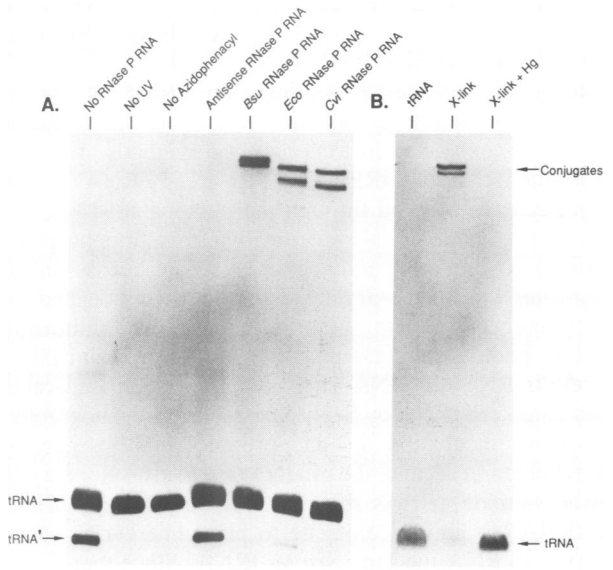

Fig. 3. Photoagent-containing tRNA cross-links to RNase P RNA. Uniformly labeled, photoagent-containing tRNA−RNase P RNA cross-links were formed as described in Materials and methods. Cross-linking reaction products were precipitated with ethanol and resolved by electrophoresis through a 4% denaturing polyacrylamide gel. (**A**) All reactions were identical except: (i) no RNase P RNA was added, (ii) the reaction was not exposed to UV light, (iii) the thio-containing tRNA was not coupled to azidophenacyl bromide, (iv) RNA complementary to *B.subtilis* RNase P RNA was added instead of RNase P RNA, (v) *B.subtilis* RNase P RNA was added, (vi) *E.coli* RNase P RNA was added and (vii) *C.vinosum* RNase P RNA was added. (**B**) Uniformly labeled tRNA was cross-linked to *B.subtilis* RNase P RNA. The conjugate species were isolated from unreacted tRNA by Sephadex G-200 chromatography (in 100 mM NaCl, 10 mM Tris, pH 7.1, 1 mM EDTA, 10 mM DTT, 0.1% SDS), and then incubated in the presence (lane 3) or absence (lane 2) of phenylmercuric acetate as described in Materials and methods. The products were then resolved by electrophoresis through a 4% denaturing polyacrylamide gel.

photoaffinity agent and UV light; however, it is not dependent upon, and indeed is suppressed by, the presence of RNase P RNA. We believe that this more rapidly migrating band results from intramolecular cross-linking in the tRNA, creating a circular RNA. The 5′- and 3′-ends of tRNAs are base-paired, therefore, in the photoagent-containing tRNA the azidophenacyl group is very close to the 3′-end (Kim *et al.*, 1973). The putatively circular species migrates more slowly than linear, mature $tRNA^{Phe}$ in higher percentage (> 8%) polyacrylamide gels (data not shown), which is consistent with the notion of intramolecular cross-linking. Anomolous behaviour of RNA in denaturing polyacrylamide gels of different concentrations is characteristic of non-linear RNAs (Sänger *et al.*, 1976). The suppression of the formation of this putatively circular species by RNase P RNA presumably relates to the nature of the interaction between the tRNA and the enzyme. The suppression may indicate that RNase P RNA partly unwinds the tRNA, separating the 5′- and 3′-ends; or, we think more likely, that the enzyme simply immobilizes the reactive group, preventing reaction except with RNase P RNA or solvent.

It was important to determine that the cross-links formed with the photoagent-containing tRNA indeed involve the RNase P RNAs and that the cross-linking occurs through the photoagent. Previous studies (Guerrier-Takada *et al.*,

1989) have shown that RNase P RNA and pre-tRNA, even without a cross-linking agent, form cross-links at low efficiency when exposed to UV light. In order to minimize such photoagent independent cross-links, we carried out irradiations at wavelengths > 300 nm (λ_{max} of the arylazide is 302 nm) as explained in Materials and methods. We also determined that the tRNA and RNase P RNA in the conjugates are coupled through the photoagent.

Since organomercurials specifically cleave thiophosphate linkages (Neumann and Smith, 1967), cross-linked conjugates due to the arylazide should be dissociated by phenylmercuric acetate. In order to test this, uniformly labeled, photoagent-containing tRNA was cross-linked to *B.subtilis* RNase P RNA, and conjugates were separated from unreacted tRNA by Sephadex G-200 chromatography. Following incubation in the presence or absence of phenylmercuric acetate, the products were resolved on a 4% polyacrylamide gel. As shown in Figure 3B, exposure of the tRNA-containing conjugate to phenylmercuric acetate releases an RNA the same size as tRNA. Since there is no detectable cross-linked species following organomercurial treatment, the cross-linking must be due solely to the azidophenacyl group. This same experiment was performed with labeled RNase P RNA and unlabeled photoagent-containing tRNA, with the same result: labeled RNase P RNA was quantitatively released from the conjugate by treatment with phenylmercuric acetate (data not shown). This result also indicates that there is no observable intramolecular cross-linking within the RNase P RNA.

We tested the RNase P activity of cross-linked complexes. If the photoagent-containing tRNA occupies the active site of RNase P RNA, it is expected that the conjugates should lack activity. Indeed, isolated conjugates containing *B.subtilis* RNase P RNA proved > 90% inactive (data not shown). Residual activity could be accounted for by some free RNase P RNA in gel-purified conjugates. Approximately 10% of the RNase P RNA is released from the conjugate species during purification. This reproducible result indicates instability of some of the cross-links and is not unprecedented (Wower *et al.*, 1989). We do not know if the labile bond(s) is at the site of insertion of the nitrene, at the thiophosphate linkage, or elsewhere. We conclude, however, that the cross-linked conjugates lack detectable RNase P activity. Additionally, if the conjugates are dissociated with phenylmercuric acetate, all the RNase P activity is restored (data not shown).

Analysis of RNase P RNA – tRNA conjugates by primer extension

Primer extension by reverse transcriptase (RT) from avian myeloblastosis virus was used to map the cross-linked sites in each of the three RNase P RNAs. RT cannot read past modifications in the RNA template, therefore, the enzyme terminates DNA synthesis at the nucleotide that immediately precedes a cross-link site in the RNase P RNA (Hagenbüchle *et al.*, 1978; Youvan and Hearst, 1982; Barta *et al.*, 1984; Inoue and Cech, 1985). Individual cross-linked conjugates were resolved by electrophoresis on a low percentage polyacrylamide gel (as in Figure 3), electroeluted and quantitated by slot blot hybridization (Materials and methods). Primer extension by RT was then carried out using an end-labeled primer complementary to the 3′-terminal sequences of the RNase P RNAs, as detailed in Materials and methods.

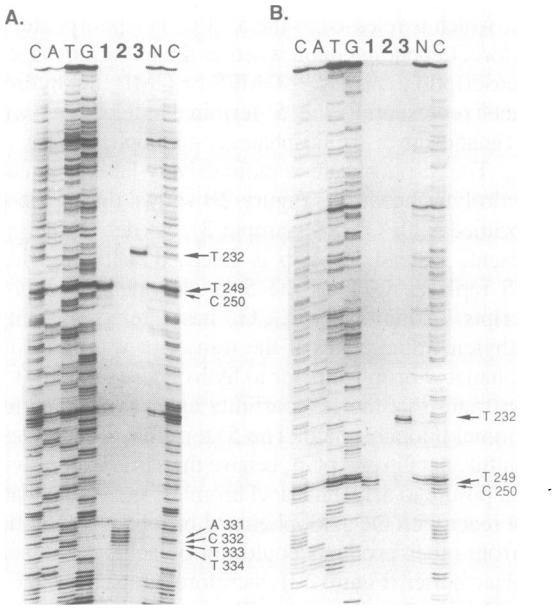

Fig. 4. Primer extension analysis of *E.coli* RNase P RNA–tRNA conjugates. Unlabeled *E.coli* RNase P RNA–*E.coli* $tRNA^{Phe}$ conjugates were prepared, purified and quantitated as described in Materials and methods. Primer extension by AMV reverse transcriptase was carried out using ^{32}P-end-labeled primer complementary to the 3′-terminal sequences of the RNase P RNAs (detailed in Materials and methods), and the products resolved on a 8% polyacrylamide sequencing gel. (**A**) Dideoxynucleotide-terminated sequencing reactions were carried out using unmodified RNase P RNA as the template (lanes C, A, T and G). Lanes 1, 2 and 3 contain primer extension products from the three different conjugate species, 1, 2 and 3 (text). Lane N contains products that result from reverse transcription of unmodified RNase P RNA. Equal amounts (10 ng) of RNase P RNA were added to each primer extension reaction in lanes 1, 2, 3 and N. (**B**) The same reaction products were resolved on a 6% polyacrylamide sequencing gel. The arrows indicate positions at which RT procession is halted by cross-links. The letter indicates the comigrating dideoxynucleotide-terminated product and the number denotes the nucleotide position of that residue in the RNase P RNA.

Figure 4A shows the results of a primer extension analysis of the *E.coli* RNase P RNA cross-linked to *E.coli* $tRNA^{Phe}$ containing the photoagent. In order to identify the sites of cross-linking, dideoxynucleotide-terminated sequencing reactions were carried out using unmodified RNase P RNA as the template (lanes C, A, T, G) and analyzed in parallel with the primer extensions on the conjugates. Lanes denoted 1, 2 and 3 contain primer extension products from the three conjugates. Conjugate 1 migrates most slowly and conjugate 3 most rapidly in a 3.5% gel (Figure 3). Lane N contains products that result from reverse transcription of unmodified RNase P RNA as in the sequencing reactions, but with no dideoxynucleotides present. This lane reveals sites at which reverse transcriptase pauses or terminates, probably because of structure in the RNA template (Lane *et al.*, 1985).

With each of the cross-linked samples as templates, prematurely terminated RT products, but no full-length products, are observed. In cross-linked *E.coli* sample 1, premature termination occurs at nucleotide positions C250 and T249. (The letter indicates the comigrating dideoxynucleotide-terminated product and is therefore complementary to the RNase P RNA sequence. The number denotes the nucleotide position in the indicated RNase P RNA.) We presume that reverse transcriptase can read up

to the cross-linked residue, but it cannot read that residue itself. We therefore interpret the site of cross-linking to be at nucleotide positions A249 and A248 of the *E.coli* RNase P RNA (structure shown below). In cross-link sample 2, premature termination products are seen at positions T334, T333, C332 and A331. We therefore infer cross-linking at nucleotides A333, G332, U331 and A330 in the RNase P RNA. Similarly, cross-link sample 3 causes a premature termination product at T232, corresponding to cross-linking at nucleotide U231. The experiment in Figure 4B is identical to 4A, except that the samples were resolved on a lower percentage sequencing gel in order to resolve more clearly cross-link products 1 and 3.

The identification of the cross-linked nucleotide presumes that termination of the reverse transcriptase occurs at the site of insertion of the nitrene. However, since tRNA occupies the active site of the RNase P RNA in the conjugates used as templates for the primer extension, it was possible that the progress of the RT was interrupted by the bulk of the tRNA, not the site of cross-linking. In order to test this possibility, cross-linked conjugates of *B.subtilis* RNase P RNA and *E.coli* $tRNA^{Phe}$ were exposed to phenylmercuric acetate, to break the cross-link prior to the primer extension analysis. This treatment leaves the arylnitrene adduct associated with the RNase P RNA. If that adduct, not the tRNA bulk, is responsible for terminating RT procession, then primer extension results should mimic those seen with the intact conjugates. The results are shown in Figure 5A. In conjugate sample 1, early termination occurs at T246 and T245, corresponding to cross-linking at nucleotides A245 and G244 in the *B.subtilis* RNase P RNA. In sample 2, early termination products occur at nucleotides T320, C319 and T318, corresponding to cross-linking at nucleotides G319, A318 and U317. The same products are seen with (+) or without (−) prior treatment with mercury. In conjugate sample 2, a termination product at nucleotide T314 also can be seen. This product is minor relative to the total products of primer extension in that sample, however, it may represent an additional cross-link site at nucleotide U313 in the RNase P RNA. In addition, primer extension analyses using either AMV reverse transcriptase of Maloney murine leukemia virus (MMLV) reverse transcriptase produced identical termination products (data not shown). All these results indicate that the main termination products with the conjugate templates are due to the cross-link. We note, however, that this indirect analysis may not identify the actual cross-linked residue in the RNase P RNA. It is possible that the RT stops at one or more nucleotides prior to the cross-link. Moreover, it is possible that the polymerase may be able to read through some of the cross-linked nucleotides, since the nitrene inserts randomly, not necessarily on bases. For these reasons, we believe that the primer extension analyses identify a region in the RNase P RNA very near the cross-link site, not necessarily the cross-linked residue itself.

In a further effort to generalize these experiments, tRNAs from different organisms as well as different RNase P RNAs were used. As shown for example in Figure 5B, *C.vinosum* RNase P RNA−tRNA cross-links were inspected using $tRNA^{Phe}$ from both *E.coli* (E) and *Saccharomyces cerevisiae* (S). In both cases, the same three cross-linked species occurred upon irradiation. The primer extension analysis shows that the two phylogenetically diverse tRNAs cross-link to precisely the same sites in the RNase P RNA.

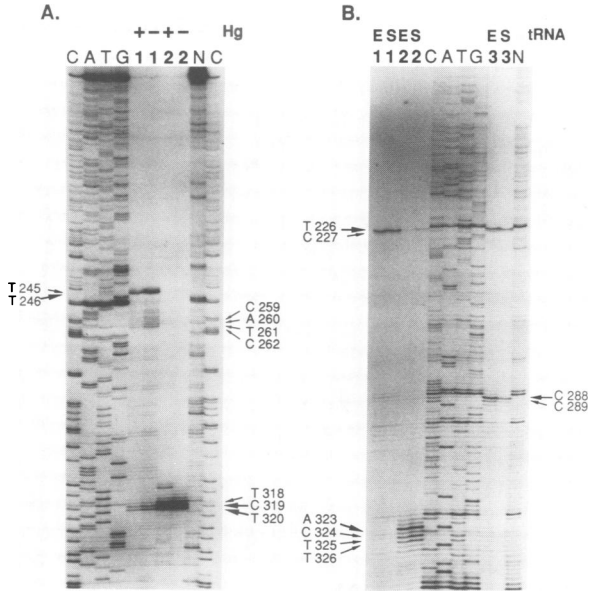

Fig. 5. Primer extension analysis of *B.subtilis* and *C.vinosum* RNase P RNA−tRNA conjugates. Unlabeled RNase P RNA−tRNA conjugates were prepared, purified and quantitated as described in Materials and methods. Primer extension by AMV reverse transcriptase was carried out as described in Materials and methods and the legend to Figure 5. (**A**) *B.subtilis* RNase P RNA−*E.coli* $tRNA^{Phe}$ conjugates (samples 1 and 2) were incubated in the presence (+) or absence (−) of phenylmercuric acetate prior to primer extension, and the products were resolved on a 8% polyacrylamide sequencing gel. (**B**) Primer extension products of *C.vinosum* RNase P RNA−*E.coli* $tRNA^{Phe}$ conjugates (denotes E) and *C.vinosum* RNase P RNA−*S.cerevisiae* $tRNA^{Phe}$ conjugates (denoted S) were resolved on a 8% polyacrylamide sequencing gel.

Conjugate 1 yields early termination products at nucleotide T226; conjugate 2 at T326, T325, C324, A323; and conjugate 3 at C289 and C288. The cross-linking data for each of the RNase P RNAs tested are summarized in Figure 6.

We note that in each of the three RNase P RNAs inspected, there is near coincidence of some primer extension products on conjugate templates with some spontaneous terminations on native templates. For instance, in the analysis of *E.coli* conjugate 1 (Figure 4), a strong spontaneous termination product (T248) occurs immediately 5′ to cross-link-induced terminations (T249 and C250). Similarly, in the analysis of *B.subtilis* conjugate 1 (Figure 5A), cross-link independent terminations occur at T251 and G250, whereas cross-link dependent terminations are observed at T246 and T245. The spontaneous terminations probably occur because RT encounters particularly stable or unusual structures within the ribozyme template.

Discussion

These experiments used mature tRNA containing a photoagent to map the active site of RNase P RNA, normally occupied by a pre-tRNA molecule. The photoagent is attached to the phosphate on which RNase P acts, and it cross-links to the ribozyme with very high efficiency (10−30%). Cross-linked conjugates containing RNase P RNA and tRNA lack detectable RNase P activity, consistent with the occupancy of the active site by the photoagent-containing tRNA. The cross-linking observed is highly

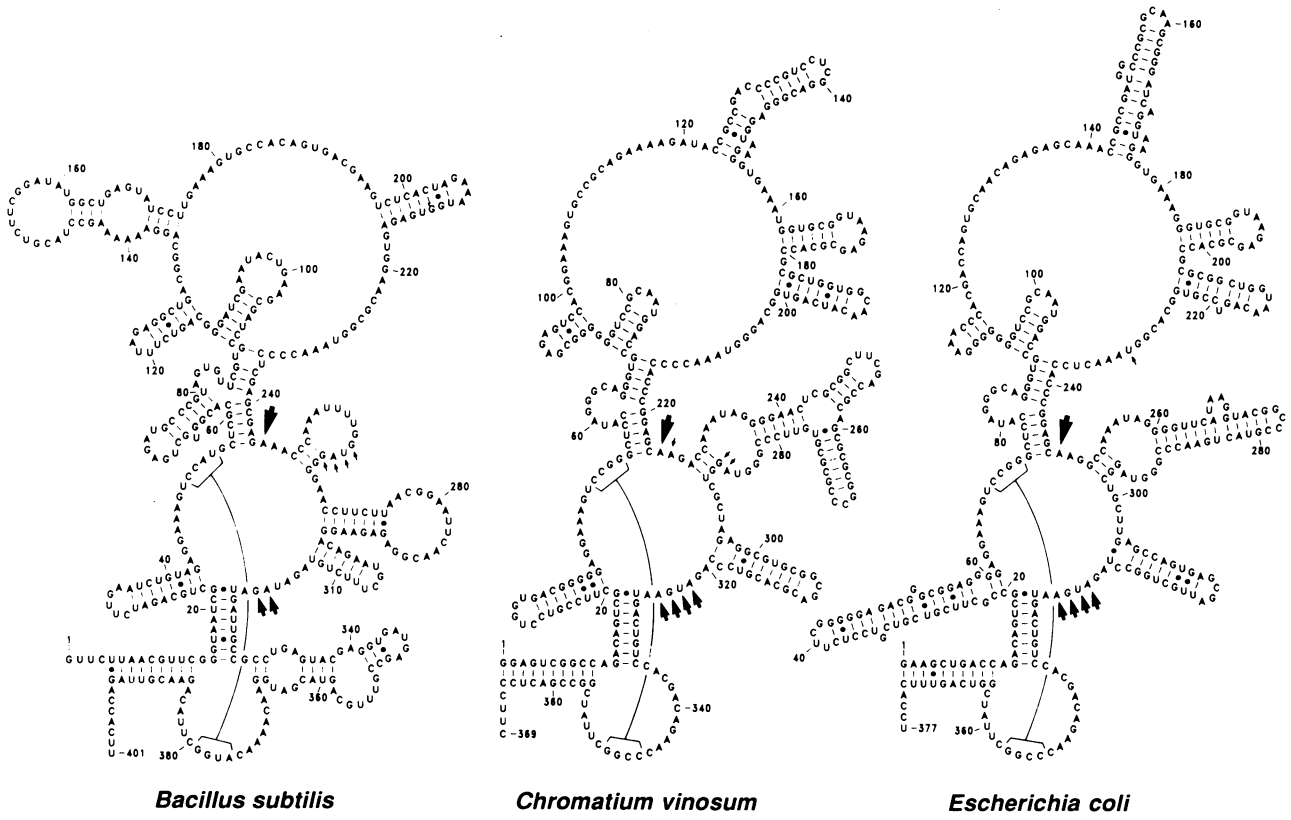

Fig. 6. Cross-linked sites in RNase P RNAs. Secondary structure models of *B.subtilis*, *C.vinosum* and *E.coli* RNase P RNAs are shown. The sites of cross-linking inferred from primer extension analysis (text) are indicated with arrows. The size of each arrow is approximately proportional to the relative amount of cross-linking seen at that residue in multiple experiments.

specific, occurring at only a few nucleotides in each of three different RNase P RNAs. Moreover, mature tRNA binds to RNase P RNA essentially as do pre-tRNA substrates (Reich, 1988). Thus, all the data indicate that mature tRNA mimicks pre-tRNA in this interaction. We believe that the cross-linking occurs at residues in the ribozyme that are adjacent to the substrate bond during the RNase P reaction.

The high efficiency of cross-linking that we observe in these reactions is probably due to the fact that, in the RNase P reaction in the absence of the protein, the rate of dissociation of tRNA product from the enzyme is slow. *In vitro*, the 'RNA-alone' reaction occurs at high ionic strength, which is thought to screen electrostatic repulsion between enzyme and substrate RNAs. One consequence of the high ionic strength is that electrostatic repulsion, which *in vivo* (at low ionic strength) would drive enzyme−product dissociation, is also screened. Therefore, *in vitro*, the complex between the RNase P RNA and the product (photoagent-containing tRNA in this case) is relatively long-lived (minutes), resulting in high efficiency of cross-linking. In contrast, little or no cross-linking is observed between photoagent-containing tRNA and RNase P holoenzyme (data not shown). The holoenzyme reaction occurs at low (physiological) ionic strength, under which conditions product dissociation from the enzyme occurs rapidly (Reich *et al.*, 1988). Therefore, cross-linking of tRNA is less likely to occur to holoenzyme under its optimal conditions than to RNase P RNA alone, at high ionic strength.

We used for these experiments RNase P RNAs from three phylogenetically disparate organisms: *B.subtilis*, *C.vinosum* and *E.coli*. These RNAs vary considerably from one another in the details of their structures, but all contain a common core of homologous sequence and secondary structure. Our motivation for carrying out these experiments with multiple RNase P RNAs was to test the generality of the findings. Particular cross-links observed in more than a single instance of RNase P RNA are likely to be the most meaningful. We also used tRNA genes from two different organisms (*E.coli* and *S.cerevisiae*) to prepare photoagent-containing tRNAs in an effort to identify idiosyncracies that might result from the use of a particular tRNA. All the cross-links were observed with both types of tRNA.

Viewed in the context of the phylogenetic structure model for eubacterial RNase P RNAs, the main regions of cross-linking are the same in each of the three RNAs inspected (Figure 6): A248−A249 (*E.coli* numbering) and A330−A333. Although these two regions are distant from one another in sequence, it is easy to imagine based on secondary structure models (Figure 6) that they are close to one another, possibly associated, in tertiary structure. It is also possible, we think less likely, that these different cross-link sites reflect different conformational states of the RNA enzymes. Because of the uniformity of the cross-linking results with all the RNAs tested, we believe that these two most prominently cross-linked regions are close to, possibly part of, the active site of RNase P. It is noteworthy that the two main cross-linked regions lie in the conserved core of the phylogenetic secondary structure, within highly conserved sequences. Such conservation is to be expected of structural elements that are important to catalytic function. However, the particular atoms to which cross-links occur (which cannot be identified in these experiments) are

unlikely to be involved specifically in catalysis. This is because reversal of the cross-links, which leaves the phenacyl nitrene adduct associated with the catalytic RNA, restores full activity to these catalytic RNAs. We would expect that modification by nitrene substitution would inactivate a catalytic chemical group in the ribozymes.

In addition to the major regions of cross-linking discussed above, there are two minor regions that are cross-linked in only one or two of the RNase P RNAs, and to lesser extent than the major regions. In *E.coli* RNase P RNA, minor cross-linking occurs in most experiments to U231; in *B.subtilis* and *C.vinosum* RNAs, some cross-linking occurs respectively at positions G293–G296 and A295–G296 (Figure 5A). These minor sites of cross-linking are located in the phylogenetically conserved core structure of the RNAs, as are the main cross-links. However, the fact that the minor cross-links occur in only one or two of the RNase P RNAs suggests that nucleotides involved may not be intimately associated with the substrate phosphate.

Guerrier-Takada *et al.* have shown that UV irradiation of *E.coli* RNase P RNA–tRNA complexes results in direct cross-linking of a nucleotide near the mature terminus of tRNA to residue C92 in RNase P RNA (Guerrier-Takada *et al.*, 1989). This is good evidence that residue C92 in the ribozyme participates in binding tRNA. The near invariance of the C residue at homologous positions in RNase P RNAs from other organisms (James *et al.*, 1988) and the location of that residue in the conserved core of the structure model are consistent with its importance in the RNase P reaction.

Finally, we note that the results reported here place constraints on structure models, since regions of the sequences cross-linked to the substrate phosphate are expected to be located adjacent to one another in the tertiary structure of the RNase P RNAs. The cross-linked nucleotides and their neighbors also provide attractive targets for site-directed mutagenesis in order to explore their involvement in the RNase P reaction.

Materials and methods

Preparation of guanosine 5′-monophosphorothioate

The synthesis of guanosine 5′-monophosphorothioate (GMPS) was based on the procedure of Murray and Atkinson (1968). Guanosine (Sigma, 566 mg, 2 mmol) was suspended in triethyl phosphate (Aldrich, 5 ml) at 100°C. The suspension was rapidly cooled to 0°C and mixed with thiophosphoryl chloride (Aldrich, 0.6 ml, 5.8 mmol). The suspension was stirred at 0°C for 12 h, then the resultant solution was mixed with 20 ml of 10% barium acetate and held at 20°C for 45 min. The suspension was divided into 6.4 ml portions, then 1.25 ml triethylamine and 15 ml of ethanol were added to each portion. The resulting precipitate was collected by centrifugation, washed with 70% ethanol and resuspended in 5 ml water. The barium salt of the nucleotide is insoluble in H_2O, so solution was effected by addition of 1 ml packed volumne of Dowex 50WX8(H^+, 20–50 mesh) cation exchange resin: H^+ is exchanged for Ba^{2+}. The resulting solution was neutralized by addition of NaOH, then loaded onto a column (2.5 cm × 15 cm) of diethylaminoethyl cellulose (Whatman, DE52) and eluted with a 200 ml linear gradient of 0–0.4 M ammonium bicarbonate in water. Fractions were collected and analyzed by polyethyleneimine TLC. Peak fractions were combined and evaporated at 25°C. The resulting residue was again dried by evaporation with 50 ml of ethanol, twice with 1 ml of triethylamine in 60 ml of 80% ethanol and once with 50 ml of ethanol. The final residue was dissolved in 3 ml of water. Total yield was 90 μmol. The product had a λ_{max} of 253 nm (250/260 = 1.17, 280/260 = 0.62). The product was shown to be the 5′-phosphorothioate by quantitative conversion to guanosine upon digestion with 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, Sigma).

Preparation of RNase P RNA

RNase P RNAs were prepared by transcription *in vitro* of plasmid DNA by phage T7 RNA polymerase (20 mM sodium phosphate pH 7.7, 8 mM $MgCl_2$, 2 mM DTT, 5 mM spermidine, 1 mM each ribonucleotide triphosphate; 37°C, 2 h) (Reich *et al.*, 1986). Transcripts were purified by electrophoresis through 3.5% polyacrylamide, 0.175% bisacrylamide, 8 M urea gels, viewed by UV shadow (Hassur and Whitlock, 1974) and passively eluted into 10 mM Tris–HCl pH 7, 1 mM EDTA, 100 mM NaCl, 0.1% SDS.

Preparation of photoagent-containing tRNA

GMPS-containing mature tRNA was prepared by *in vitro* transcription of plasmid DNA by phage T7 RNA polymerase (20 mM sodium phosphate pH 7.7, 15 mM $MgCl_2$, 20 mM DTT, 5 mM spermidine, 1 mM ATP, 1 mM CTP, 1 mM UTP, 0.2 mM GTP, 4 mM GMPS; 37°C, 2 h). Transcripts were purified by electrophoresis through 6% polyacrylamide, 0.3% bisacrylamide, 8 M urea gels, viewed by UV shadow and passively eluted into 10 mM Tris–HCl pH 7, 1 mM EDTA, 10 mM DTT, 0.1% SDS. Transcripts were ethanol-precipitated twice, dried *in vacuo* and resuspended in 40% methanol, 20 mM sodium bicarbonate pH 9.0, 0.1% SDS, 5 mM azidophenacyl bromide (Pierce) and incubated at room temperature for 1 h (Hixson and Hixson, 1975; Hanna and Meares, 1983). Excess azidophenacyl bromide was extracted with two volumes phenol and the photoagent-containing tRNA was recovered by ethanol precipitation.

Formation of RNase P RNA – tRNA cross-links

A typical photolabeling reaction contained 100 ng photoagent-containing tRNA and 5 μg RNase P RNA in 50–200 μl of 50 mM Tris–HCl pH 8.0, 800 mM NH_4OAc, 100 mM $MgCl_2$. The RNAs were incubated at 37°C for 5 min, then placed on ice for 2 min. The mixture was then exposed to UV light (Model UVM-57 302 nM lamp, UVP, San Gabriel, CA) at 50 mm, screened by a polystyrene filter (American Scientific Products diSPo Petri dish, D1937) for 5–60 min at 0°C. Polystyrene absorbs short wavelengths (< 300 nm). Under these conditions, no photoagent independent cross-linking could be detected. It was found that broad spectrum UV light (germicidal lamp), or short wave UV light (Model UVS-54 254 nm lamp, UVP), in the absence of a polystyrene filter, produced cross-links in the absence of the photoagent. A xenon chloride laser (308 nm) was used in some experiments and also induced high efficiency, photoagent dependent cross-linking.

Mercury-facilitated cleavage of RNase P RNA – tRNA cross-links

RNase P RNA–tRNA conjugates were ethanol precipitated and dried *in vacuo*. The RNA was resuspended in 5 μl H_2O and added to 95 μl of 10% isopropanol, 25 mM Tris–HCl pH 8.0, 0.1% SDS, saturated with phenylmercuric acetate (Aldrich) and incubated at room temperature for 30 min. Excess phenylmercuric acetate was removed by extraction with two volumes of phenol followed by extraction with two volumes of anhydrous ether. The RNAs were recovered by ethanol precipitation.

Primer extension analysis

RNase P RNA–tRNA conjugates were resolved by electrophoresis through 3.5% acrylamide, 0.175% bisacrylamide, 8 M urea, TBE (90 mM Tris–HCl pH 8.0, 90 mM boric acid, 2 mM EDTA), 1 mm thick slab gels. Conjugate species were identified by ethidium bromide staining (2 μg/ml), and electroeluted (Model UEA, International Biotechnologies, New Haven, CT) in 20 mM Tris–HCl pH 8.0, 5 mM NaCl, 1 mM EDTA at 100 V for 15 min. The amount of RNase P RNA in each conjugate sample was determined by comparison of slot blot hybridization signals between known amounts of RNase P RNA and each cross-linked sample, using uniformly ^{32}P-labeled, partially hydrolyzed, complementary RNase P RNA (prepared by *in vitro* transcription as described, except that the reaction contained 100 μM GTP and [α-^{32}P]GTP). In a typical primer extension experiment, 1 ng of 5′-^{32}P-labeled oligonucleotide specific for the particular RNase P RNA was hybridized to 10 ng RNase P RNA–tRNA conjugate (20 mM Tris–HCl pH 8.0, 30 mM KCl, 90°C, 2 min, then slowly cooled over 10 min to room temperature). Primers used were: for *B.subtilis*, GTGGTCTAACGTTCTGT; for *C.vinosum*, CGATGAAGGGAGTCGGCC; for *E.coli*, GTAAATCAGGTGAAA. The oligonucleotides were extended by AMV reverse transcriptase (Seikaguku, 1 unit) in 50 mM Tris–HCl pH 8.0, 10 mM DTT, 10 mM $MgCl_2$, 100 μM each dNTP at 45°C for 20 min. Each dideoxynucleotide sequencing lane contained a 5-fold molar excess of RNase P RNA as compared with each cross-link lane in Figures 4 and 5. The products were resolved by electrophoresis through 8% polyacrylamide (0.4% bisacrylamide) gels containing 8 M urea in 0.5 × TBE.

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