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Substrate Shape Specificity of *E. coli* RNase P Ribozyme Is Dependent on the Concentration of Magnesium Ion

Tomoaki Ando, Terumichi Tanaka* and Yo Kikuchi

Division of Bioscience and Biotechnology, Department of Ecological Engineering, Toyohashi University of Technology, Tempaku-cho, Toyohashi, Aichi 441-8580

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The bacterial RNase P ribozyme can accept a hairpin RNA with CCA-3′ tag sequence as well as a cloverleaf pre-tRNA as substrate *in vitro*, but the details are not known. By switching tRNA structure using an antisense guide DNA technique, we examined the *Escherichia coli* RNase P ribozyme specificity for substrate RNA of a given shape. Analysis of the RNase P reaction with various concentrations of magnesium ion revealed that the ribozyme cleaved only the cloverleaf RNA at below 10 mM magnesium ion. At 10 mM magnesium ion or more, the ribozyme also cleaved a hairpin RNA with a CCA-3′ tag sequence. At above 20 mM magnesium ion, cleavage site wobbling by the enzyme in tRNA-derived hairpin occurred, and the substrate specificity of the enzyme became broader. Additional studies using another hairpin substrate demonstrated the same tendency. Our data strongly suggest that raising the concentration of metal ion induces a conformational change in the RNA enzyme.

Key words: antisense, guide DNA, hyperprocessing, magnesium, RNase P.

Ribonuclease P (RNase P) is a tRNA-processing enzyme that produces mature tRNA molecules by cleaving the tRNA precursor at the 5′-end (*1*, *2*). It is a ribonucleoprotein enzyme, consisting one RNA subunit and one or more protein subunit(s). The RNA subunit of bacterial and of some archaeal RNases P is a ribozyme, which is able to exert the RNase P activity without the protein component (*3–11*).

Two bacterial enzymes, *Escherichia coli* and *Bacillus subtilis* RNasse P, have been most extensively studied. The tertiary structure of the RNA component has not been solved, however, and therefore the studies on RNase P ribozyme have mainly been phylogenetic using mutant constructs. RNase P is metal-dependent ribozyme, and several metal-binding sites have been identified (*12–23*). On the other hand, in spite of many reports, the intrinsic role of the protein component is unclear (*3–11*).

Recently, we reported that some mature tRNAs are internally cleaved by bacterial RNase P *in vitro* (*24–39*). We denoted this unusual cleavage of tRNA as hyperprocessing, and compiled the experimental data of hyperprocessible tRNAs and extracted the criteria for the occurrence of the hyperprocessing reaction (*38*). Why does the bacterial RNase P catalyze the unusual hyperprocessing reaction to destroy mature tRNA? We knew that magnesium ion takes part in the reaction: but how? In this report, we studied the substrate recognition of the *E. coli* RNase P ribozyme with a hyperprocessible tRNA. For this study, we also developed an antisense guide DNA technique to control the substrate shape (*30*, *39*). Our data reveal how the RNase P ribozyme recognizes the target RNA dependently on the metal ion concentration.

*To whom correspondence to should be addressed. Fax: +81-532-44-6929, E-mail: tanakat@eco.tut.ac.jp

Previously, we reported that the cloverleaf structure of some mature tRNAs is not always stable, and that it is denatured to form a double-hairpin-like structure *in vitro* (*24*, *39*). The denatured tRNA can be efficiently detected through the bacterial RNase P reaction when the 3′-half of the denatured tRNA forms an alternative extended hairpin structure (*38*). Human $tRNA^{Tyr}$ is one such hyperprocessible tRNA *in vitro* (*34*, *37*). In the presence of 5 mM magnesium ion, the usual processing reaction of the pre-tRNA to produce the mature tRNA by the *E. coli* RNase P is observed but the hyperprocessing is never observed. On the other hand, both the processing and the hyperprocessing reactions are observed with more than 10 mM magnesium ion (*37*). The previous results indicated that the human $tRNA^{Tyr}$ can provide two types of substrates for the RNase P, the cloverleaf and the hairpin RNAs, in the presence of higher magnesium ion concentrations. Under the standard reaction conditions, the content of hyperprocessible hairpin-folded RNA is thought to be minor, we have chosen this tRNA as a probe in this study, and applied the guide DNA technique to the RNase P reaction as described below.

MATERIALS AND METHODS

Preparation of RNAs and Other Chemicals—E. coli ribonuclease P RNA was prepared by *in vitro* transcription from the pGEM-3Z-derived plasmid DNA with T7 RNA polymerase according to the method described previously (*24*).

The human tyrosine tRNA precursor was prepared by *in vitro* transcription from the pGEM-3Z-derived plasmid DNAs as described previously (*34*, *37*). Synthetic antisense guide DNAs were purchased from Funakoshi Corp.: 5′-TGTAC AGTCC TCCGC TCTAC CAGCT GAGCT ATCGA-3′ (for "STN-DNA"), 5′-CTCAG ACTTT TAATC TGAGT CTACA GTCCT CCGCT CTACC AGCTG AGCTA

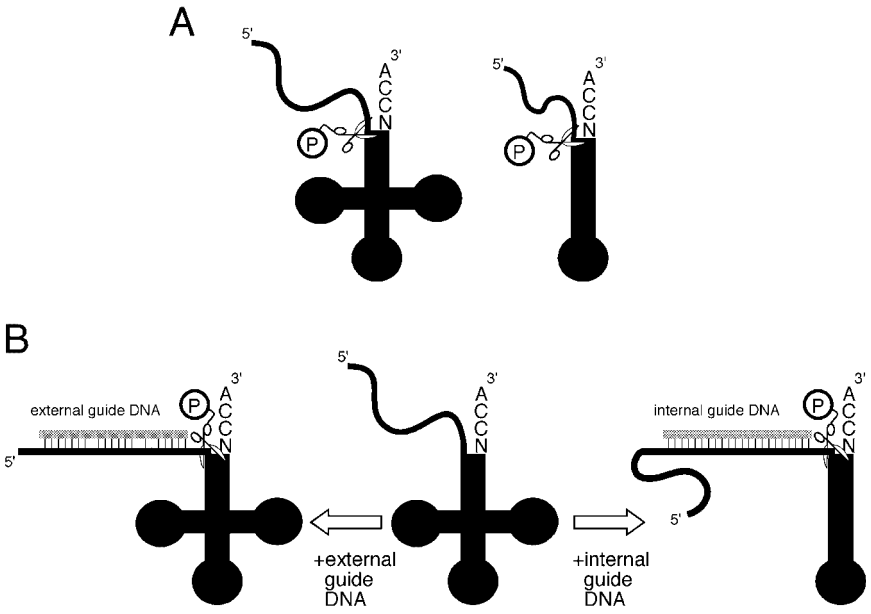

Fig. 1. **Schematic representation of RNase P reaction and guide DNA technique.** (A) Schematic representation of substrate recognition by the bacterial RNase P. The bacterial enzymes recognize the tRNA precursor in the cloverleaf structure (left) as well as the hairpin RNA with CCA-3′ tag sequence (right) as substrate. The scissor represents the cleavage site in the RNA. (B) The guide DNA technique used in this study. The antisense DNA to the 5′-leader region of the pre-tRNA, indicated as external guide DNA, enhances the usual processing reaction of pre-tRNA by the bacterial RNase P (left). On the other hand, the antisense DNA to the internal region of certain tRNA, indicated as internal guide DNA, contributes to the destruction of the cloverleaf structure of tRNA. If the 3′-half of the denatured tRNA can form an alternative extended hairpin, the RNA is further cleaved by the enzyme (right; denoted as hyperprocessing; the criteria are described in the Ref. 38).

TCGA-3′ (for "5end bulge DNA"), 5′-TCTAC AGTCC TCCGCT CCTCA GACTT TTAAT CTGAG ACCAG CTGAG CTATC GA-3′ (for "Midbulge-DNA"), 5′-ACCAT CTACA GTCCT CCGCT CTACC AGCTG AGCTA TCGA-3′ (for "ACCA-tag DNA"), and 5′-AACCA GCGAC CTAAG GATCT ACAGT CCTCC GCTCT ACCA-3′ (for "Dest-DNA").

The *Drosophila* pre-tRNA^{Val} was also prepared by *in vitro* transcription from the pGEM-3Z-derived plasmid DNAs as described previously (*30*, *39*). The hairpin RNA derived from the *Drosophila* tRNA^{Val} was prepared by *in vitro* transcription from the synthetic partially double-stranded DNAs containing the T7 promoter sequence: 5′-TGGTG TTTTC GCCCG GGTTC GAACC GGGCG AAAAC ATCAT GAACG AATTC GCCCT ATAGT GAGTC GTATT ACA-3′ and 5′-TGTAA TACGA CTCAC TATAG GGCGA ATTCG TTCAT GATGT TTTCG-3′.

Cleavage Assay of RNAs—E. coli RNase P RNA and the tRNA precursor were prepared by *in vitro* transcription with T7 RNA polymerase ('TT7 RNA polymerase', Toyobo) using cleaved DNA templates. The human tRNA^{Tyr} precursor was labeled at the 3′-end with [5′-^{32}P]pCp and T4 RNA ligase as described previously. The RNase P reaction of human pre-tRNA^{Tyr} in the presence or absence of guide DNA was done under standard conditions (0.72 μM *E. coli* RNase P RNA, 60.3 nM pre-tRNA^{Tyr}, 5 μM each guide DNA, 5%[w/v] polyethylene glycol, 100 mM NH_4Cl, 50 mM Tris-HCl, at pH 8.0, 37°C; for 1 h) and in the presence of 0, 2.5, 5, 10, 20, or 40 mM Mg^{2+} and the reaction products were developed on 20% PAGE and analyzed as described previously (*24*). The *Drosophila* tRNA^{Val} precursor and the hairpin RNA were labeled at the 5′-end with [^{32}P-g]ATP and T4 polynucleotide kinase as described previously. The RNase P reaction of the *Drosophila* pre-tRNA^{Val} and the hairpin RNA was also done under standard conditions (0.78 μM *E. coli* RNase P RNA, 320 nM pre-tRNA^{Val} or 25.3 nM hairpin RNA, 5%[w/v] polyethylene glycol, 100 mM NH_4Cl, 50 mM Tris-HCl, at pH 8.0, 37°C; for 2 h) and in the presence of 0, 5, 10, 20, or 40 mM Mg^{2+} and the reaction products were developed on 20% PAGE and analyzed as described previously (*24*).

RESULTS AND DISCUSSION

The Guide DNA Technique to Switch the Shape of RNA—We developed an antisense guide DNA technique to promote and to control the RNase P reaction. Of the two types of antisense guide DNA, external and internal, the external guide DNA is the antisense DNA hybidizable to the 5′-leader region of the pre-tRNA (Refs. *30* and *39*; Fig. 1B, left). In the bacterial RNase P reaction, the presence of the external guide DNA enhances the RNase P reaction. This phenomenon means that the bacterial RNase P accepts the RNA/DNA duplex at the 5′-leader region, and that the presence of the guide DNA enhances the correct cloverleaf folding of the pre-tRNA. The antisense external guide DNA technique is applicable to efficient preparation of mature tRNA from the pre-tRNA.

In this study, we used the antisense internal guide DNA technique to destroy the cloverleaf shape of the target tRNA. Our previous report suggested that the application of the internal guide DNA to the hyperprocessible tRNA would efficiently destroy the cloverleaf shape of tRNA (Ref. *30*; Fig. 1B, right). In the hyperprocessible tRNA, the 3′-half of the tRNA can form an alternative extended hairpin. The antisense DNA, the internal guide DNA, to the 5′-upstream region of the cleavage site is expected to enhance the extended hairpin formation. The design of the antisense internal guide DNAs and the target region of the tRNA are shown in Fig. 2. Four guide DNAs are designed to hybridize to the same region of the tRNA, U^4-A^{37}, and one guide DNA is designed to hybridize to another region, U^{17}-U^{55}.

The results of the RNase P reaction in the absence and presence of the internal guide DNA are shown in Fig. 3. The results in the absence of guide DNA showed that the processing reaction of pre-tRNA occurred with magnesium ion above 2.5 mM ("Mat" in Fig. 3A, lanes 2–6), and the hyperprocessing occurred above 10 mM ("Hyp1" and

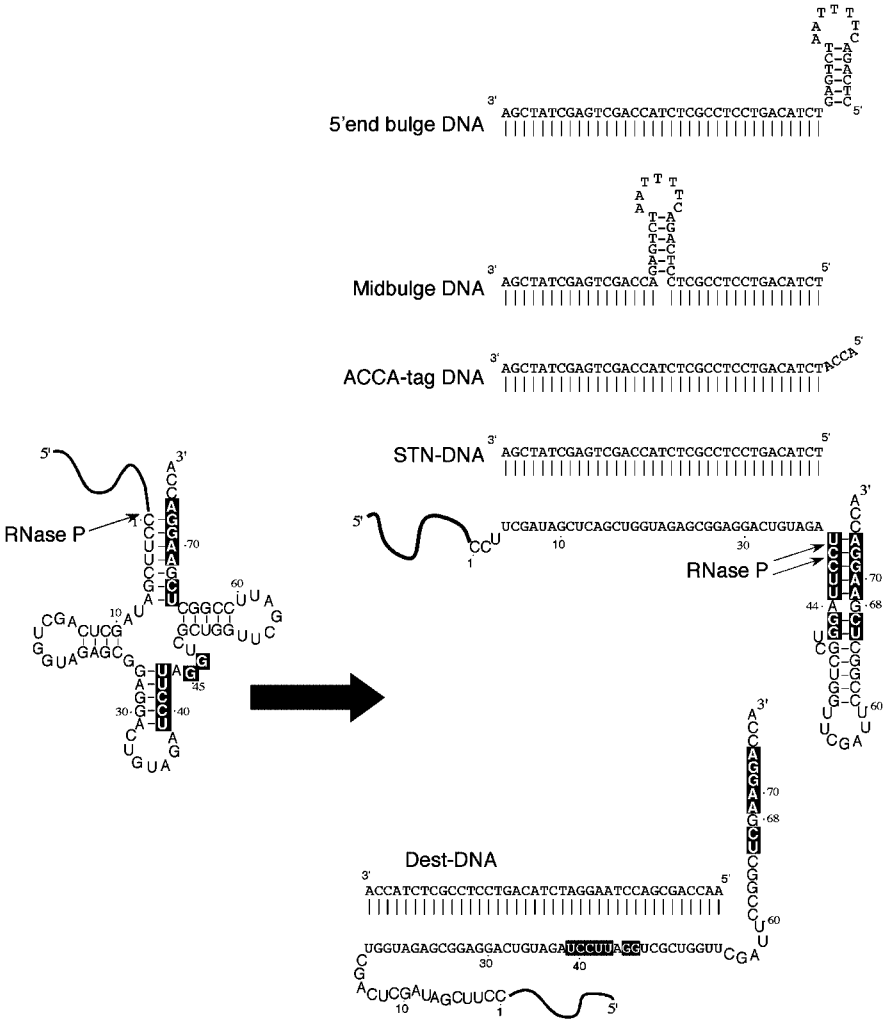

Fig. 2. **Guide DNAs in used this study and the target region in the pre-tRNA.** Five guide DNAs were used for this study: "STN-DNA" is a 35-mer DNA hybridizable to the U^{4}-A^{37} region of the target tRNA, "ACCA-tag DNA" has an additional four-base sequence at the 5′-end of the "STN-DNA", "5′ end bulge DNA" has an additional short hairpin at the 5′-end of the "STN-DNA," "Midbulge DNA" has the same hairpin sequence as "5′ end bulge DNA," but located in the middle of the sequence; and "Dest-DNA" is a 35-mer DNA hybridizable to the U^{17}-U^{55} region of the target tRNA. The regions U^{39}-G^{45} and U^{66}-A^{73} of the target pre-tRNA, which contribute to the formation of the hyperprocessible extended hairpin, are indicated by boxes. The cleavage sites by the RNase P are shown by arrows. See Fig. 3.

"Hyp2" in lanes 4–6). In this case, most RNA molecules in the reaction mixture were thought to be in the cloverleaf structure, because the processing reaction requires the cloverleaf shape of pre-tRNA. On the other hand, the results in the presence of the guide DNA "STN-DNA" showed that the processing, the maturation reaction of pre-tRNA, was hardly observed ("Mat" in Fig. 3A, lanes 10–14). Considering both the lack of the processed product and the amount of hyperprocessed products obtained, the pre-tRNA was thought to be almost completely denatured by the guide DNA to form the hyperprocessible hairpin structure at every concentration of magnesium ion. Comparison of the data in the absence and in the presence of the guide DNA indicates that the hyperprocessing of tRNA absolutely requires the magnesium ion at 10 mM or more. The difference in the content of hyperprocessed products, we think, comes from the difference in the content of hairpin-fold RNA in the reaction mixture in the absence or presence of the guide DNA.

The other two guide DNAs, "5end bulge DNA" (Fig. 3A, lanes 17–22) and "ACCA-tag DNA" (lanes 33–38), gave similar results to "STN-DNA": the processing reaction of pre-tRNA was completely inhibited at every concentration of magnesium ion, and the hyperprocessed products were observed in the presence of 10 mM magnesium ion or more. These data indicated that the additional nucleotide at the 5′-end of the guide DNA, supposed to be located near the cleavage site, did not affect the RNase P reaction. The results with "Midbulge DNA" as guide DNA (lanes 28–30) were slightly different from those with 'STN-DNA': the mature product was observed at 2.5 and 5 mM in this case (lanes 26 and 27). The results suggested that the presence of nucleotide mismatch in the guide DNA affects the hybridization to the target tRNA, which allows the pre-tRNA to re-fold to the canonical cloverleaf structure: we suppose that the disappearance of mature-size product above 10 mM magnesium (lanes 28–30) is due to the hyperprocessing of the newly formed mature tRNA.

The results with "Dest-DNA" (Fig. 3A, lanes 41–46) showed that the guide DNA to the region U^{17}-U^{55} completely inhibited the hyperprocessing reaction and also reduced the efficiency of the processing reaction.

The above data indicated that the guide DNA technique is applicable to select the cleavage site and to control the cleavage efficiency of the hyperprocessible tRNA.

Substrate Shape Recognition of E. coli RNase P Ribozyme Varies with Magnesium Ion Concentration—The *E. coli* RNase P ribozyme exhibitied the enzyme activity at low magnesium ion concentration of above 2.5 mM (Fig.

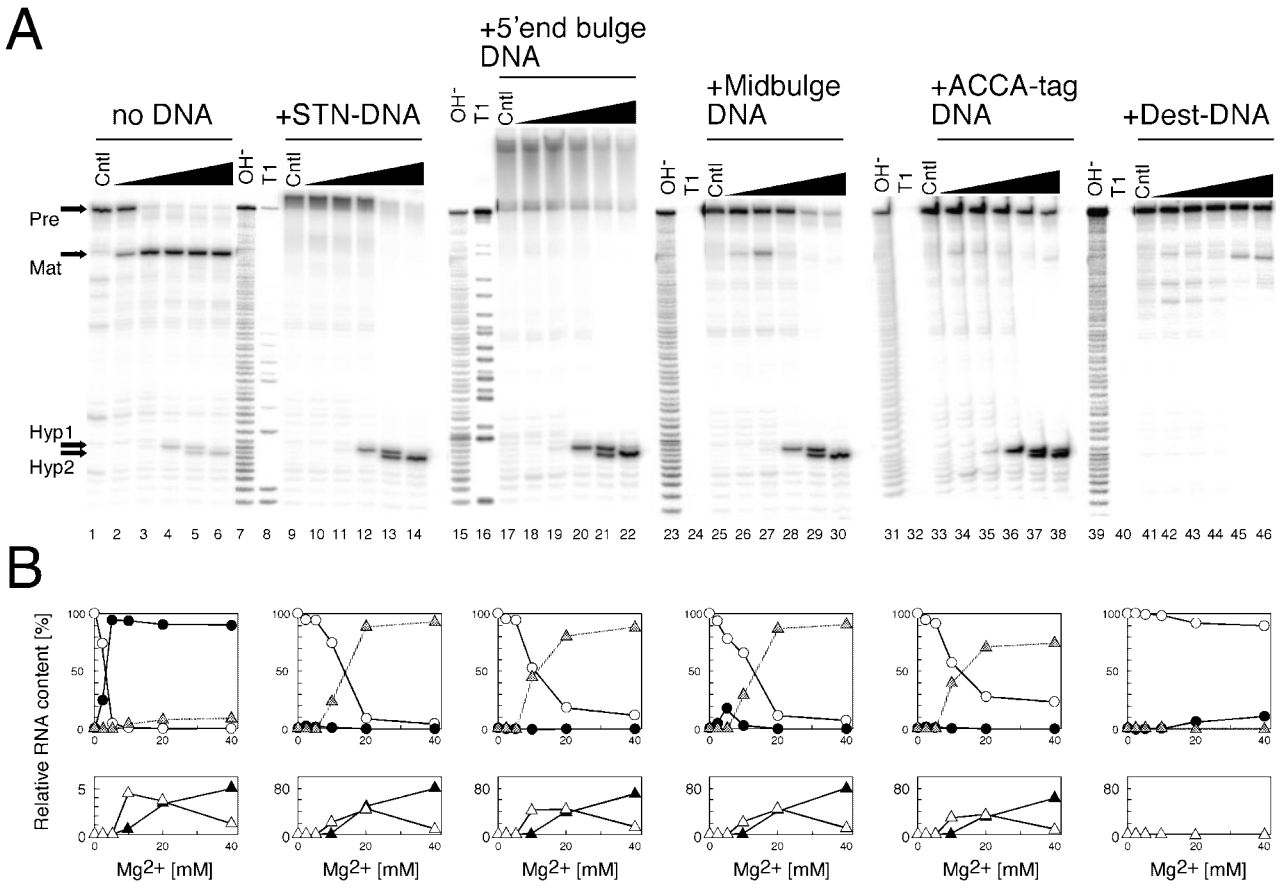

Fig. 3. **RNase P reaction of tRNA with guide DNA.** The RNase P reaction of the human pre-tRNA^{Tyr} was done in the absence or presence of each guide DNA. The *E. coli* RNase P ribozyme was used as an enzyme. The reaction was done with various concentrations of magnesium ion (0, 2.5, 5, 10, 20, or 40 mM $MgCl_2$) in a mixture containing 50 mM Tris-HCl, 5% (w/v) polyethylene glycol, 100 mM NH_4Cl, 0.72 μM *E. coli* RNase P ribozyme, 60.3 nM human pre-tRNA^{Tyr}, and 5 μM guide DNA, at pH 8.0, 37°C, for 1 h. (A) PAGE analysis of the products developed on 20% PAGE. "Pre," "Mat," "Hyp1," and "Hyp2" on the left side of the photo represent the pre-tRNA, mature tRNA, product-1, and product-2, respectively. "Cntl," lane 2, represents the products of the control reaction done in the absence of magnesium ion. 'Cntl', lanes 9, 17, 24, 33, and 41, represents the products of the control reaction done in the absence of the enzyme but in the presence of 40 mM magnesium ion and each guide DNA. "OH$^-$" and "T1" represent the partially alkaline hydrolyzed size marker and the nuclease T1 hydrolyzed size marker, respectively. (B) The relative RNA content was analyzed from the photo. Open circles, pre-tRNA; closed circles, mature tRNA; hatched triangles, hyperprocessed products (sum of product-1 and –2) (above); open triangles, product-1 ("Hyp1"); and closed triangles, product-2 ("Hyp2") (below).

3), which is consistent with other reports (*12–23*). With 5 mM magnesium ion, the ribozyme cleaved only cloverleaf shape pre-tRNA; the ribozyme could not cleave the hairpin RNA formed by the binding of guide DNA. With above 10 mM magnesium ion, hyperprocessed products appeared, which indicated the ribozyme accepted and cleaved the hairpin RNA as a substrate. Considering the results in the absence of guide DNA (Fig. 3A, lanes 1–6), these data indicate that the substrate specificity of this ribozyme, in the view of recognition of the shape of RNA, becomes broader and looser as the concentration of magnesium ion is raised. Moreover, the substrate cleavage pattern of the ribozyme changed at the magnesium ion concentration of 20 mM: the ribozyme produced two cleaved products both in the absence and in the presence of guide DNA (lanes 5, 13, 21, and 37), except in the presence of 'Dest-DNA' as guide DNA (lane 45). With 40 mM magnesium ion, the major cleavage site in hyperprocessing shifted from the longer one (cleavage at U^{39}-C^{40} bond) to the shorter one (cleavage at C^{40}-C^{41}). In the presence of 'Dest-DNA', the mature product was observed at above 20 mM (lanes 45 and 46), which suggests that the refolded acceptor-stem of the pre-tRNA was recognized as sub-

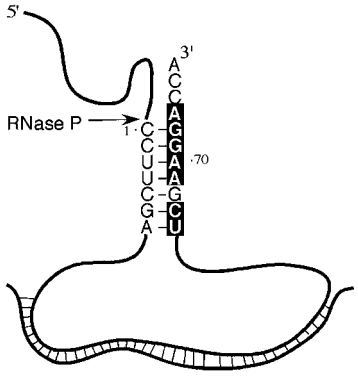

Fig. 4. **Possible structure of target tRNA with Dest-DNA.** See text.

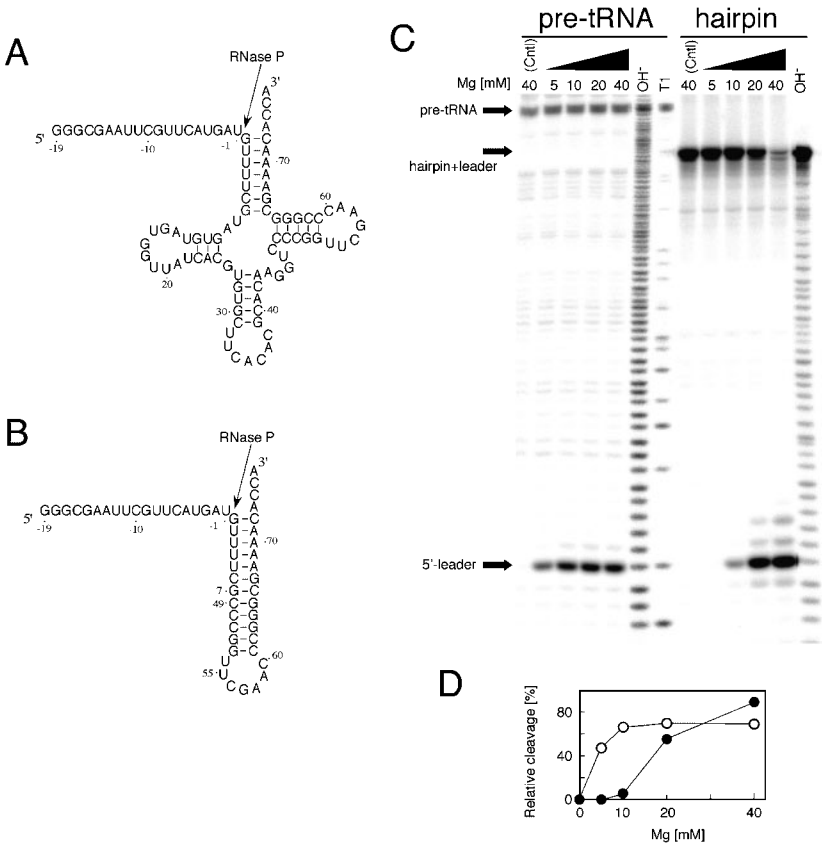

Fig. 5. **RNase P reaction of pre-tRNA and hairpin substrates.** (A) *Drosophila* pre-tRNA-^{Val}. (B) Hairpin RNA derived from the tRNA^{Val}. The cleavage site by the *E. coli* RNase P ribozyme is indicated by an arrow. (C) The RNAs were labeled at the 5′-end and developed on PAGE after the RNase P reaction at 37°C for 120 minutes (0.78 μM *E. coli* RNase P RNA, 320 nM pre-tRNA or 25 nM hairpin RNA, 5–40 mM $MgCl_2$, 100 mM NH_4Cl, 5% (w/v) polyethylene glycol, 50 mM Tris-HCl; pH 8.0; in 10 μL scale reaction). "OH−" and "T1" represent the partially alkaline hydrolyzed size marker, and the nuclease T1 partially hydrolyzed size marker, respectively. "Cntl" represents the control reaction in the absence of the enzyme but in the presence of 40 mM magnesium ion. (D) The relative values of the cleaved RNA were plotted against the magnesium concentration. Open and closed circles represent the values of the pre-tRNA and the hairpin substrates, respectively.

strate by the enzyme (the putative secondary structure is shown in Fig. 4). Considering that no hyperprocessed products were observed in the presence of the "Dest-DNA," we suppose that the guide DNA still bound to the pre-tRNA substrate in the reaction, destroying the hyperprocessible hairpin structure formation.

Higher Magnesium Ion Was also Required to Cleave a Hairpin Substrate—The above results showed that the more than 10 mM magnesium ion was required for the cleavage of the hairpin RNA substrates derived from tRNA. Mutational analyses of the hyperprocessed tRNAs in our previous studies showed that the hairpin formation of the 3′-half of the tRNA is required for the hyperprocessing reaction (*24*, *34*), which strongly suggests that the hairpin formation of the 3′-half the of the target tRNA also occurred in this case. To confirm the magnesium ion concentration-dependence of the hairpin recognition by the ribozyme, we did additional experiments, because of the technical difficulties of the direct observation of the hairpin formation of the target RNA: we prepared a hairpin RNA and examined the RNase P reaction. The artificial RNA has complementary regions to form a 12-base length helix derived from the tRNA^{Val} and is expected to form a hairpin (Fig. 5B). The results, shown in Fig. 5, indicated that the magnesium ion-dependence of the RNase P reaction was same as the results in Fig. 3: the hairpin cleavage by the ribozyme was observed in the presence of 10 mM magnesium ion or more. The shift of the substrate shape recognition of the *E. coli* ribozyme is shown schematically in Fig. 6.

The Reason for the Shift of the Substrate Recognition of the RNase P Ribozyme—The bacterial RNase P ribozyme has several metal binding sites, of which some contribute

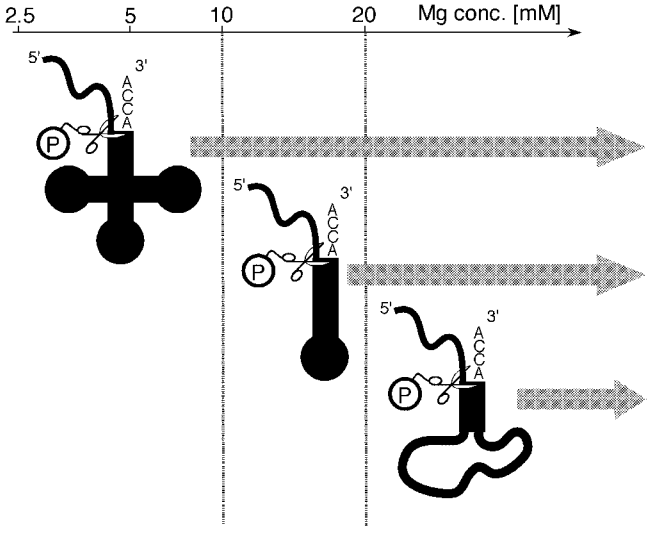

Fig. 6. **Schematic representation of the substrate recognition of *E. coli* RNase P ribozyme with various concentrations of magnesium ion.** At a certain concentration of magnesium ion, the *E. coli* RNase P RNA exhibits enzymatic activity to cleave pre-tRNA in cloverleaf shape. With 10 mM magnesium ion or more, the ribozyme also recognizes a hairpin RNA with a CCA-3′ tag sequence as a substrate. At above 20 mM magnesium ion, the cleavage site wobbling by the enzyme in tRNA-derived hairpin occurs, and the substrate specificity of the enzyme becomes broader.

to the correct folding of the RNA, and some contribute to the formation of the catalytic center (*12–23*). In the RNA enzyme, the cation contributes to countervail the negative surface charge of the nucleotide, which reduces the barrier to interaction between the ribozyme and the substrate RNA. In case of the *E. coli* ribozyme, the presence of low magnesium ion concentration of around 2.5–5 mM is necessary for efficient folding of RNA structure and the efficient acceptance of pre-tRNA substrate in the cloverleaf folding. Our data indicated that raising the concentration of magnesium ion to above 10 mM enables the ribozyme to accept the non-cloverleaf RNAs. Moreover, the higher concentration of metal ion, we suppose, induces a conformational change in the ribozyme that retsuls in the cleavage site shift, as observed at around 20 mM magnesium ion (Fig. 3). This explanation can also account for the hyperprocessing of tRNA. The tRNA structure can be denatured by a high concentration of magnesium ion (*40*), and some tRNA can be denatured *in vitro* (*24–39*). In some tRNAs, the 3′-half of the denatured tRNA can form an alternative extended hairpin, and the newly formed hairpin can be recognized by the bacterial enzyme at higher magnesium concentration. Our data showed that the occurrence of the hyperprocessing due to the substrate shape recognition of the enzyme depended on the magnesium ion concentration.

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REFERENCES

1. Pace, N.R. and Brown, J.W. (1995) Evolutionary perspective on the structure and function of ribonuclease P, a ribozyme. *J. Bacteriol.* **177**, 1919–1928
2. Altman, S., Kirsebom, L.A., and Talbot, S. (1995) Recent studies of RNase P inn tRNA Structure, biosynthesis, and functions. (Söll, D. and RajBhandary, U., eds.) pp. 67–78, American Society for Microbiology Press, Washington, DC
3. Pannucci, J.A., Haas, E.S., Hall, T., Harris, J.K., and Brown, J.W. (1999) RNase P RNAs from some archaea are catalytically active. *Proc. Natl Acad. Sci. USA* **96**, 7803–7808
4. True, H.L. and Celander, D.W. (1998) Protein component contributes to active site architecture for eukaryotic ribonuclease P. *J. Biol. Chem.* **273**, 7193–7196
5. Kim, M., Park, B.H., and Lee, Y. (2000) Effects of terminal deletions in C5 protein on promoting RNase P catalysis. *Biochem. Biophys. Res. Commun.* **268**, 118–123
6. Park, B.H., Lee, J.H., Kim, M., and Lee, Y. (2000) Effects of C5 protein on *Escherichia coli* RNase P catalysis with a precursor $tRNA^{Phe}$ bearing a single mismatch in the acceptor stem. *Biochem. Biophys. Res. Commun.* **268**, 136–140
7. Niranjanakumari, S., Kurz, J.C., and Fierke, C.A. (1998) Expression, purification and characterization of the recombinant ribonuclease P protein component from *Bacillus subtilis*. *Nucleic Acids Res.* **26**, 3090–3096
8. Kurz, J.C., Niranjanakumari, S., and Fierke, C.A. (1998) Protein component of *Bacillus subtilis* RNase P specifically enhances the affinity for precursor-$tRNA^{Asp}$. *Biochemistry* **37**, 2393–2400
9. Niranjanakumari, S., Stams, T., Crary, S.M., Christianson, D.W., and Fierke, C.A. (1998) Protein component of the ribozyme ribonuclease P alters substrate recognition by directly contacting precursor tRNA. *Proc. Natl Acad. Sci. USA* **95**, 15212–15217
10. Stams, T., Niranjanakumari, S., Fierke, C.A., and Christianson, D.W. (1998) Ribonuclease P proteins structure: evolutionary origins in the translational apparatus. *Science* **280**, 752–755
11. Reich, C., Olsen, G.J., Pace, B., and Pace, N.R. (1988) Role of the protein moiety of ribonuclease P, a ribonucleoprotein enzyme. *Science* **239**, 178–181
12. Fang, X.W., Pan, T., and Sosnick, T.R. (1999) Mg^{2+}-depedent folding of a large ribozyme without kinetic traps. *Nat. Struct. Biol.* **6**, 1091–1095
13. Loria, A., Niranjanakumari, S., Fierke, C.A., and Pan, T. (1998) Recognition of a pre-tRNA substrate by *Bacillus subtilis* RNase P holoenzyme. *Biochemistry* **37**, 15466–15473
14. Lazard, M. and Meinnel, T. (1998) Role of base G_{-2} of pre-$tRNA^{fMet}$ in cleavage sit selection by *Escherichia coli* RNase P *in vivo*. *Biochemistry* **37**, 6041–6049
15. Kufel, J. and Kirsebom, L.A. (1998) The P15-loop of *Escherichia coli* RNase P RNA is an autonomous divalent metal ion binding domain. *RNA* **4**, 777–788
16. Oh, B.K., Frank, D.N., and Pace, N.R. (1998) Participation of the 3′-CCA of tRNA in the binding of catalytic Mg^{2+} ions by ribonuclease P. *Biochemistry* **37**, 7277–7283
17. Warnecke, J.M., Fürste, J.P., Hardt, W.D., Erdmann, V.A., and Hartmann, R.K. (1996) Ribonuclease P RNA is converted to a Cd^{2+}-ribozyme by a single Rp-phosphorothianate modification in the precursor tRNA at the RNase P recognition site. *Proc. Natl Acad. Sci. USA* **93**, 8924–8928
18. Beebe, J.A., Kurz, J.C., and Fierke, C.A. (1996) Magnesium ions are required by *Bacillus subtilis* ribonuclease P RNA for both binding and cleaving precursor $tRNA^{Asp}$. *Biochemistry* **35** 10493–10505
19. Smith, D. and Pace, N.R. (1993) Multiple magnesium ions in the ribonuclese P reaction mechanism. *Biochemisty* **32**, 5273–5281
20. Pan, T. and Jakacka, M. (1996) Multiple substrate binding sites in the ribozyme from *Bacillus subtilis* RNase P. *EMO J.* **15**, 2249–2255
21. Fang, X., Pan, T., and Sosnick, T.R. (1999) A thermodynamic framework and cooperativity in the tertiary folding of a Mg^{2+}-dependent ribozyme. *Biochemistry* **38**, 16840–16846
22. Christian, E.L., Kaye, N.M., and Harris, M.E. (2000) Helix 4 is a divalent metal ion binding site in the conserved core of the ribonuclease P ribozyme. *RNA* **6**, 511–519
23. Fang, X., Littrell, K., Yang, X., Henderson, S.J., Siefert, S., Thiyagarajan, P., Pan, T., and Sosnick, T.R. (2000) Mg^{2+}-dependent compaction and folding of yeast $tRNA^{Phe}$ and the catalytic domain of the *B. subtilis* RNase P RNA determined by small-angle X-ray scattering. *Biochemistry* **39**, 11107–11113
24. Hori, Y., Baba, H., Ueda, R., Tanaka, T., and Kikuchi, Y. (2000) *In vitro* hyperprocessing of *Drosophila* tRNAs by the catalytic RNA of RNase P. *Eur. J. Biochem.* **267**, 4781–4788
25. Kikuchi, Y., Sasaki, N., and Ando-Yamagami, Y. (1990) Cleavage of tRNA within the mature tRNA sequence by the catalytic RNA of RNase P: Implication for the formation of the primer tRNA fragment for reverse transcription in *copia* retrovirus-like particles. *Proc. Natl Acad. Sci. USA*. **87**, 8105–8109
26. Kikuchi, Y. and Sasaki, N. (1992) Hyperprocessing of tRNA by the catalytic RNA of RNase P. *J. Biol. Chem.* **267**, 11972–11976
27. Kikuchi, Y., Sasaki-Tozawa, N., and Suzuki, K. (1993) Artificial self-cleaving molecules containing of an tRNA precursor and the catalytic RNA of RNase P. *Nucleic Acids Res.* **21**, 4685–4689
28. Kikuchi, Y. and Suzuki-Fujita, K. (1995) Synthesis and self-cleavage reaction of a chimeric molecule between RNase P-RNA and its model substrate. *J. Biochem.* **117**, 197–200
29. Kikuchi, Y. (1996) RNase P as hyperprocessing enzyme: A model for formation of a biologically functional tRNA fragment. *Mol. Biol. Rep*. **22**, 171–175
30. Tanaka, T., Baba, H., Hori, Y., and Kikuchi, Y. (2001) Guide DNA technique reveals that the protein component of bacterial

ribonuclease P is a modifier for substrate recognition. *FEBS Lett.* **491**, 94–98
31. Hori, Y., Tanaka, T., and Kikuchi, Y. (2000) The catalytic RNA of RNase P from *Escherichia coli* cleaves *Drosophila* 2S ribosomal RNA in vitro: a new type of naturally occurring substrate for the ribozyme. *FEBS Lett.* **472**, 187–190
32. Hori, Y., Bichenkova, E.V., Wilton, A.N., El-Attug, M.N., Sadat-Ebrahimi, S., Tanaka, T., Kikuchi, Y., Araki, M., Sugiura, Y., and Douglas, K.T. (2001) Synthetic inhibition of the processing of pretransfer RNA by the ribonuclease P ribozyme: Enzyme inhibitors which act by binding to substrate. *Biochemistry* **40**, 603–608
33. Tanaka, T., Inui, O., Dohi, N., Okada, N., Okada, H., and Kikuchi, Y. (2001) Is your ribozyme design really correct?: A proposal of simple single turnover competition assay to evaluate ribozymes. *Biosci. Biotechnol. Biochem.* **65**, 1636–1644
34. Ando, T., Tanaka, T., Hori, Y., Sakai, E., and Kikuchi, Y. (2001) Human tyrosine tRNA is also internally cleavable by *E. coli* ribonuclease P RNA ribozyme *in vitro*. *Biosci. Biotechnol. Biochem.* **65**, 2798–2801
35. Tanaka, T. and Kikuchi, Y. (2001) Origin of the cloverleaf shape of transfer RNA—the double-hairpin model: Implication for the role of tRNA intron and the long extra loop. *Viva Origino* **29**, 134–142
36. Hori, Y., Sakai, E., Tanaka, T., and Kikuchi, Y. (2001) Hyperprocessing reaction of tRNA by *Bacillus subtilis* ribonuclease P ribozyme. *FEBS Lett.* **505**, 337–339
37. Ando, T., Tanaka, T., Hori, Y., and Kikuchi, Y. (2002) Kinetic analysis on hyperprocessing reaction of human tyrosine tRNA by *E. coli* ribonuclease P ribozyme. *Biosci. Biotechnol. Biochem.* **66**, 1967–1971
38. Tanaka, T., Kondo, Y., Hori, Y., and Kikuchi, Y. (2002) Another cut for lysine tRNA: Application of the hyperprocessing reaction reveals another stabilization strategy in metazaon lysine tRNAs. *J. Biochem.* **131**, 839–847
39. Tanaka, T., Hori, Y., and Kikuchi, Y. (2002) Guide DNA technique in bacterial ribonuclease P reaction for effective processing of tRNA precursor. *Biotechnol. Appl. Biochem.* **36**, 85–88
40. Helm, M., Brule, H., Degoul, F., Cepanec, C., Leroux, J.P., Giege, R., and Florentz, C. (1998) The presence of modified nucleotides is required for cloverleaf folding of a human mitochondrial tRNA. *Nucleic Acids Res.* **26**, 1636–1646