Identification of Nucleotide Residues Essential for RNase P Activity from the Hyperthermophilic Archaeon Pyrococcus horikoshii OT3

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Ribonuclease P (RNase P) is involved in the processing of the 5' leader sequence of precursor tRNA (pre-tRNA). We have found that RNase P RNA (PhopRNA) and five proteins (PhoPop5, PhoRpp21, PhoRpp29, PhoRpp30, and PhoRpp38) reconstitute RNase P activity with enzymatic properties similar to those of the authentic ribozyme from the hyperthermophilic archaeon Pyrococcus horikoshii OT3. We report here that nucleotides A40, A41, and U44 at helix P4, and G269 and G270 located at L15/16 in PhopRNA, are, like the corresponding residues in Escherichia coli RNase P RNA (M1RNA), involved in hydrolysis by coordinating catalytic Mg$^{2+}$ ions, and in the recognition of the acceptor end (CCA) of pre-tRNA by base-pairing, respectively. The information reported here strongly suggests that PhopRNA catalyzes the hydrolysis of pre-tRNA in approximately the same manner as eubacterial RNase P RNAs, even though it has no enzymatic activity in the absence of the proteins.

Key words: Archaea; Pyrococcus horikoshii; precursor tRNA (Pre-tRNA); ribozyme; ribonuclease P RNA (RNase P RNA)

Although the functionality of RNase P remains almost the same from bacteria to humans, the chemical composition and enzymatic properties of this enzyme differ in three phylogenetic domains of life: Eubacteria, Archaea, and Eukarya. Eubacterial RNase P is composed of a catalytic RNA and a single protein subunit, and in the presence of a high concentration of Mg$^{2+}$, the eubacterial RNase P RNA itself can hydrolyze pre-tRNA in vitro. In contrast, eukaryotic RNase P RNAs comprise a single RNA moiety and as many as 10 proteins, and the RNA component itself has no catalytic activity in vitro. As for archaean RNase P, the presence of several protein components associated with their RNAs has been suggested. It was reported that some archaean RNase P RNAs have no enzyme activity in the cleavage of pre-tRNA, though some have catalytic activity in the presence of unusually high salt concentrations. Earlier, we found in reconstitution experiments that RNase P RNA (PhopRNA) in the hyperthermophilic archaeon Pyrococcus horikoshii OT3, like eukaryotic counterparts, is deficient in function and cooperates with five protein subunits (PhoPop5, PhoRpp21, PhoRpp29, PhoRpp30, and PhoRpp38) in substrate recognition and/or catalysis.

Ever since Altman and co-workers discovered that the Escherichia coli RNase P RNA (M1 RNA) is a ribozyme, biochemical and structural studies have focused on eubacterial RNase P RNAs. These studies have provided evidence that the P1-P4 multihelix junction plays a crucial role in the optimization of Mg$^{2+}$ interactions important for catalysis. In particular, nucleotides A65 and A66 at J3/4 and helix P4 and the pro-Rp and pro-Sp non-bridging phosphate oxygens at A67 in helix P4 were assigned as binding sites for Mg$^{2+}$ ions required for catalysis by M1 RNA. Furthermore, M1 RNA is known to be involved in substrate recognition, interacting with both the mature tRNA domain and pre-tRNA.

Although RNase Ps in Archaea and Eukarya contain additional proteins, their RNAs retain an essential core of conserved sequence and secondary structure. It is thus likely that RNase P RNAs are directly involved in catalytic function. However, investigation of archaean and eukaryotic RNase P RNAs in vitro has been limited by the lack of catalytic activity of the RNA alone. Since we have achieved the reconstitution of the P. horikoshii RNase P, we examined here the involvement of conserved nucleotide residues in PhopRNA in RNase P activity. In this study, we identified A40, A41, and U44 as essential nucleotide residues for catalysis of PhopRNA. In addition, G269 and G270, located at L15/16, are
Materials and Methods

Materials. Five RNase P proteins (PhoPop5, PhoRpp21, PhoRpp29, PhoRpp30, and PhoRpp38), wild-type PhopRNA, and $^{32}$P-labeled pre-tRNA$^{3yr}$ in *P. horikoshii* were prepared as described previously. All other chemicals were of analytical grade for biochemical use.

Site-directed mutagenesis. Site-directed mutagenesis of PhopRNA and pre-tRNA$^{3yr}$ was done on DNA templates in plasmid pUC19 using a QuickChange Site-Directed Mutagenesis Kit (Stratagene) as suggested by the manufacturer. All mutant RNA genes were sequenced to confirm the presence of intended mutations only. The template plasmids coding for RNA mutants from other organisms, as described in reference 22. The site-specific mutants are denoted with the wild-type residue given first, followed by the position number, and the new residue ([e.g., the PhopRNA mutant in which G269 in the wild-type is replaced with C is referred to as pRNA-G269C]).

Results and Discussion

Figure 1A shows the secondary structure of PhopRNA. It consists of 329 nucleotides, and includes 16 double-stranded regions, referred to as P1–P16, as presented in the RNase P database (http://jwbrown.mbio.ncsu.edu/RNaseP/home.html). Characteristic differences in secondary structure between archaean RNA (PhopRNA) and eubacterial RNA (M1 RNA) are that archaean PhopRNA lacks P13, P14, and P18 in M1 RNA and acquires the terminal helical structures P12.1 and

Fig. 1. The Secondary Structure of PhopRNA from *P. horikoshii* OT3.

A, The secondary structure of PhopRNA from *P. horikoshii* is shown. B, Secondary structure of J3/4 and helix P4 in M1 RNA and PhopRNA is shown. Nucleotide residues at helix P4 are boxed.
Table 1. Characterization of RNase P Activity of the PhoρpRNA Mutants

<table>
<thead>
<tr>
<th>pRNA</th>
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<th>pRNA</th>
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<tbody>
<tr>
<td>A37</td>
<td>G39</td>
<td>A41</td>
<td>U43</td>
</tr>
<tr>
<td>U (100%)</td>
<td>U (100%)</td>
<td>U (24%)</td>
<td>C (98%)</td>
</tr>
<tr>
<td>C (100%)</td>
<td>C (99%)</td>
<td>C (38%)</td>
<td>G (72%)</td>
</tr>
<tr>
<td>G (73%)</td>
<td>A (100%)</td>
<td>G (12%)</td>
<td>A (96%)</td>
</tr>
<tr>
<td>pRNA-G38</td>
<td>pRNA-A40</td>
<td>pRNA-G42</td>
<td>pRNA-U44</td>
</tr>
<tr>
<td>U (94%)</td>
<td>U (15%)</td>
<td>U (71%)</td>
<td>C (75%)</td>
</tr>
<tr>
<td>C (60%)</td>
<td>C (32%)</td>
<td>C (92%)</td>
<td>G (26%)</td>
</tr>
<tr>
<td>A (99%)</td>
<td>G (41%)</td>
<td>A (76%)</td>
<td>A (71%)</td>
</tr>
</tbody>
</table>

RNase P activities of the in vitro reconstituted mixture were assayed at 65°C for 5 min using 32P-labeled P. horikoshii pre-rRNA\textsuperscript{59} as a substrate as described in “Materials and Methods.” The activities of the reconstituted particles shown in parentheses were expressed relative to that of the reconstituted RNase P containing wild-type PhoρpRNA (100%).

Essential nucleotide residues for catalysis

It is known that nucleotide bases A65 and A66 and non-bridging phosphate oxygens at A67 in M1 RNA are involved in the stabilization of catalytic Mg\textsuperscript{2+} ions.\textsuperscript{16} In addition, a bulged U69 at helix P4 plays a role in maintaining the helical geometry important for interaction with Mg\textsuperscript{2+} ions.\textsuperscript{17} Although PhoρpRNA differs from M1 RNA in that it has no nuclease activity in the absence of proteins, its nucleotide sequence and secondary structure at J3/4 and helix P4 are highly homologous with those of M1 RNA, as presented in Fig. 1B. The possible secondary structure of each mutant RNA is presented in Table 1. First, we investigated whether a bulged helical structure corresponding to J3/4 and helix P4 in M1 RNA plays an equivalent role in the activity of PhoρpRNA. For this purpose, eight nucleotide residues, from A37 to U44, located at J3/4 and helix 4 in PhoρpRNA, were in turn replaced with three other nucleotides. The 24 resulting mutant PhoρpRNAs were synthesized by in vitro transcription, as described in “Materials and Methods,” and then characterized in terms of RNase P activity in the presence of five proteins (Table 1). The RNase P activities of the mutants, in which A40 and A41 were replaced with the three other residues, ranged from 12% to 41% of that of the wild-type RNA. The double mutation of A40 and A41 abolished RNase P activity (data not shown). In addition, replacing U44 with G significantly reduced RNase P activity (26%). In contrast, mutations of other residues had only a modest effect on RNase P activity: the reconstituted RNase P containing the other mutant PhoρpRNAs retained 60%–100% of RNase P activity as compared to wild-type RNase P activity. The results indicated that A40, A41, and U44 are, like their counterparts, A65, A66, and possibly U69 respectively, involved in the nuclease activity of PhoρpRNA.

Evaluation of a bulged structure at U44

It has been reported that the presence of a bulged residue at U69 in M1 RNA is important for interaction with metal ions.\textsuperscript{10} The present study indicated that U44 located at helix 4 in PhoρpRNA, which possibly corresponds to U69 in M1 RNA, is essential for RNase P activity. It is, however, known that archaeal PhoρpRNAs have a neighboring U residue at position 43. To exclude the possibility that U43 instead of U44 bulges at helix P4, we prepared four mutant RNAs (U44-I, U44-II, U44-III, and U44-IV) with distinct pairs at this helix. The possible secondary structure of each mutant RNA is presented in Fig. 2. The mutant RNAs were prepared, and their RNase P activity was analyzed in the presence of the proteins. The results showed that a double mutant, U44-II, in which U43 and G311 are replaced with G and C respectively and U44 is presumed to be unpaired, exhibited nuclease activity comparable to the wild-type (Fig. 2). In contrast, the other three mutants, in which the nucleotide at position 44 interacts with Watson-Crick complementarity, showed reduced RNase P activity, ranging from 6% to 21% of that of the wild type (Fig. 2). It is thus likely that the nucleotide U44 is unpaired and forms a bulged structure at helix P4 in PhoρpRNA.

Mg\textsuperscript{2+} dependency of RNase P activity of the mutants

The present findings indicate that nucleotides A40, A41, and U44 in PhoρpRNA are essential for RNase P activity. It is known that the corresponding residues, A65, A66, and U69, are involved in the stabilization of Mg\textsuperscript{2+} ions, which play an essential role in RNase P activity of M1 RNA. However, these residues are not conserved in PhoρpRNA. Therefore, the nucleotides involved in the stabilization of Mg\textsuperscript{2+} ions in PhoρpRNA may differ from those in M1 RNA. Further studies are needed to determine the specific roles of the nucleotides in PhoρpRNA.
activity. To examine further the functional equivalence of A40, A41, and U44 in PhopRNA to A65, A66, and U69 in M1 RNA, we analyzed the Mg$^{2+}$-dependency of mutant PhopRNAs at different concentrations of Mg$^{2+}$. As Fig. 3A and B show, that the RNase P activity of all mutant RNAs except for the double mutant was restored at a higher concentration of Mg$^{2+}$ ions. The double mutant pRNA-A40U/A41U, in which both nucleotides A40 and A41 were replaced with U residues, had little RNase P activity even at a higher concentration of Mg$^{2+}$ ions. This result strongly suggests that A40, A41, and U44 in PhopRNA are functionally equivalent to A65, A66, and U69 in M1 RNA and are involved in the stabilization of Mg$^{2+}$ ions. It is probably that the double mutation of A40 and A41 resulted in a drastic destabilization of the helical structure, and consequently the helical geometry important for the binding of Mg$^{2+}$ at helix P4 could no longer be maintained.

Essential nucleotide residues for pre-tRNA recognition

Among eubacterial RNase PPs, base pairings between RNase P RNA and its substrate are essential for substrate recognition. Previous studies have investigated some such possible base pairings. In M1 RNA, it was found that G292, G293, and U294 in the L15/16 domain, which are well conserved among the bacterial RNase P RNAs, base pair with C75, C74, and R73 in the acceptor stem of the pre-tRNA RCCA sequence.19) In addition, U(−1) in the pre-tRNA 5' leader sequence is assumed to base pair with A248 in the J5/15 domain.20) The nucleotides in the L15/16 and J5/15 domains are conserved in PhopRNA: G269, G270, U271, and A227 in PhopRNA correspond to G292, G293, U294, and A248 in M1 RNA respectively (Fig. 4A). Accordingly, we investigated whether these nucleotides in PhopRNA are, like their counterparts in M1 RNA, involved in

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Fig. 3. Magnesium Rescue of Mutations at J3/4 and Helix P4 in PhopRNA.
A. The RNase P activity assays were carried out at Mg$^{2+}$ concentrations of 0, 2.5, 5, 10, 25, and 50 mM, as described in “Materials and Methods,” and the cleavage products were resolved on 15% acrylamide/8 M urea/TBE gels and visualized by autoradiography. a, b, c, d, e, and f indicate RNase P activities of the reconstituted RNase P containing wild-type PhopRNA, pRNA-A40U, pRNA-A41U, pRNA-A40U/A41U, pRNA-U44G, and U44-IV respectively. B, Graphic representation of the pre-tRNA cleavage seen in A. a, wild type; o, A40U; □ A41U; ■ A40U/A41U; △, U44G; ○, U44-IV. P, M, and L indicate pre-tRNA$^{3′}$, mature tRNA$^{3′}$, and 5’-leader fragment respectively.

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Fig. 4. Nucleotide Residues Involved in Substrate Recognition of P. horikoshii RNase P.
A. The secondary structure of L15/16, J5/15, and pre-tRNA is shown. Predicted interaction of nucleotides at L15/16 and J5/15 in PhopRNA with those at the 3′- and 5′-ends respectively of pre-tRNA$^{3′}$ is shown by dashed lines. The arrow indicates the processing site of pre-tRNA$^{3′}$ by P. horikoshii RNase P.25) B, Characterization of the enzyme activity of the in vitro reconstituted RNase P containing either wild-type PhopRNA or mutant PhopRNAs. The RNase P activities of the in vitro reconstituted mixture were assayed, and the ribozyme activities by the reconstituted particles containing the mutant PhopRNAs were expressed relative to that of the reconstituted RNase P containing wild-type PhopRNA (100%).
substrate recognition. Thus G269, G270, and U271 in the L15/16 domain and C226, A227, and A228 in the J5/15 domain were in turn replaced with three other nucleotides. The 18 resulting mutant RNAs were synthesized by in vitro transcription, as described in “Materials and Methods,” and then characterized with respect to RNase P activity in the presence of five proteins. As a result, the five mutants with changes to G269 and G270 in the L15/16 domain showed reduced RNase P activities (less than 20%), while the U271 mutants and one mutant, pRNA-G270C, exhibited modestly reduced activities (of more than 40%) (Fig. 4B). As for mutants altered at J5/15, the A228 mutants and single mutants, pRNA-C226G and -A227U, for C226 and A227, showed decreased RNase P activity, while the other mutations had little effect on RNase P (Fig. 4B). These results suggest that nucleotide residues in the L15/16 and J5/15 domains in Pho RNA are modestly involved in RNase P activity, possibly base pairing with nucleotide residues at the acceptor end and in the 5′ leader sequence respectively.

To test this assumption, we examined whether compensatory changes in the pre-tRNA$_{\text{Tyr}}$ could restore RNase P activity for mutant Pho RNAs. First, the mutant pre-tRNA$_{\text{C135G}}$, in which C135 in the pre-tRNA$_{\text{Tyr}}$ was replaced with G, was prepared and then in turn it was incubated with PhoRNA-G269C, -G269A, and -G269U in the presence of five proteins. Among these three PhoRNA mutants, pRNA-G269C significantly restored RNase P activity toward pre-tRNA$_{\text{C135G}}$, while the other two mutants, pRNA-G269A and -G269U, showed similar RNase P activity toward both the wild-type pre-tRNA$_{\text{Tyr}}$ and the mutant pre-tRNA$_{\text{C135G}}$ (Table 2). The result suggests that restoration of the RNase P activity of the mutant pRNA-G269C is due to re-base pairing between the G at position 135 in the mutant pre-tRNA$_{\text{Tyr}}$ and the C at position 269 in the mutant PhoRNA. Similarly, replacement of C134 in pre-tRNA$_{\text{Tyr}}$ with A restored the RNase P activity of pRNA-G270U (Table 2); the reconstituted RNase P containing the mutant pRNA-G270U exhibited the highest RNase P activity among the three pRNA-G270 mutants. In this analysis, the mutants, pRNA-G270A and -G270C, exhibited increased RNase P activity toward the pre-tRNA$_{\text{Tyr}}$ mutant (pre-tRNA-C134A), showing 110%–120% RNase P activity (Table 2). Although we have no explanation for this finding at present, it is likely that the mutation of C134 in pre-tRNA$_{\text{Tyr}}$ by A causes a conformational change that makes a scissile bond in the mutant pre-tRNA$_{\text{Tyr}}$ more susceptible to RNase P than in wild-type pre-tRNA$_{\text{Tyr}}$. On the other hand, the mutation of A133 in pre-tRNA$_{\text{Tyr}}$ did not rescue the RNase P activities of the three Pho RNA mutants, pRNA-U271C, -U271A, and -U271G (Table 2). These results suggest that G269 and G270, like the corresponding residues G292 and G293 in M1 RNA, are probably involved in recognition of pre-tRNA, base pairing with the acceptor end C135 and C134 respectively in pre-tRNA$_{\text{Tyr}}$. The present results, however, suggest that U271 in PhoRNA, unlike the corresponding residue U294 in M1 RNA, has no direct interaction with A133 in pre-tRNA$_{\text{Tyr}}$.

Finally, the nucleotide U at position 58 in pre-tRNA$_{\text{Tyr}}$ was replaced with C, and the resulting mutant pre-tRNA$_{\text{U58C}}$ was individually incubated with the PhoRNA mutants pRNA-A227G, -A227C, and -A227U in the presence of five proteins (Table 2). The mutant pRNA-A227G as well as the other two mutants, pRNA-A227C and -A227U, showed increased RNase P activity (Table 2). This result suggests that A227 in PhoRNA, unlike the corresponding nucleotide A248 in M1 RNA, does not base pair with the U at position 58 in pre-tRNA$_{\text{Tyr}}$. Similar results were obtained from assays with mutant pre-tRNA-U58C and PhoRNA mutants altered at nucleotides C226 and A228 (Table 2). Hence it is probable that the nucleotides at J5/15 in PhoRNA participate in RNase P activity by non-base pairing with the nucleotide at the 5′ leader sequence of pre-tRNA.

Conclusions

The present study indicates that the bulge stem-loop structure containing J3/4 and helix P4 is involved in the interaction with Mg$^{2+}$ ions important for the catalysis of PhoRNase P. In addition, nucleotides G269 and G270 in the L5/15 domain in PhoRNA are probably involved in the recognition of pre-tRNA$_{\text{Tyr}}$, possibly

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**Table 2. Ribozyme Activities of Mutant PhoRNAs toward the Wild-Type Pre-tRNA and Its Mutants**

<table>
<thead>
<tr>
<th>PhoRNA</th>
<th>pre-tRNA</th>
<th>PhoRNA</th>
<th>pre-tRNA</th>
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</thead>
<tbody>
<tr>
<td>G269C</td>
<td>C135 (38%)</td>
<td>G270 (140%)</td>
<td>C226A</td>
</tr>
<tr>
<td>G269A</td>
<td>C135 (39%)</td>
<td>G135 (60%)</td>
<td>C226G</td>
</tr>
<tr>
<td>G269U</td>
<td>C135 (36%)</td>
<td>G135 (60%)</td>
<td>C226U</td>
</tr>
<tr>
<td>G270A</td>
<td>C134 (38%)</td>
<td>A134 (185%)</td>
<td>A227G</td>
</tr>
<tr>
<td>G270A</td>
<td>C134 (36%)</td>
<td>A134 (120%)</td>
<td>A227C</td>
</tr>
<tr>
<td>G270C</td>
<td>C134 (50%)</td>
<td>A134 (110%)</td>
<td>A227U</td>
</tr>
<tr>
<td>U271C</td>
<td>A133 (55%)</td>
<td>G133 (65%)</td>
<td>A228G</td>
</tr>
<tr>
<td>U271I</td>
<td>A133 (57%)</td>
<td>G133 (63%)</td>
<td>A228C</td>
</tr>
<tr>
<td>U271G</td>
<td>A133 (60%)</td>
<td>G133 (60%)</td>
<td>A228U</td>
</tr>
</tbody>
</table>

RNase P activities of the in vitro reconstituted mixture were assayed at 65°C for 20 min, as described in “Materials and Methods.” Numbers in parentheses are the ribozyme activities relative to that of the reconstituted RNase P containing wild-type PhoRNA (100%). The PhoRNAs underlined are mutants that restored RNase P activity toward the pre-tRNA mutants containing compensatory changes, as shown in boxes.
base pairing with the nucleotides at the acceptor end in pre-tRNA. In contrast, A227 at 15/15 exhibited no direct Watson-Crick interaction with the nucleotide U at position 58 in pre-tRNA$^X$. It is hence concluded that archaeal RNase P RNA PhopRNA catalyzes the hydrolysis of pre-tRNA$^X$ in approximately the same manner as eubacterial RNase P RNAs, even though it has no enzymatic activity in the absence of the proteins.

During preparation of this report, Kikovska et al. reported that eukaryotic RNase P RNAs mediate cleavage of tRNA precursors.$^{24}$ It is hence now established that the RNA-based catalytic activity of RNase P is preserved in three phylogenetic domains of life during evolution.

**Acknowledgments**

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**References**