The P3 domain of *E. coli* ribonuclease P RNA can be truncated and replaced

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Abstract We prepared some truncated and replaced P3 mutants of *Escherichia coli* RNase P RNA, and used them to examine the RNase P ribozyme and holoenzyme reactions of a pre-tRNA substrate. The results indicated that mutations in the P3 domain did not affect the cleavage site selection of the pre-tRNA substrate, but did affect the efficiency of cleavage of the substrate. Results of stepwise truncation of the P3 domain and its replacement by the TAR sequence showed that the P3 domain of the *E. coli* RNase P was able to be truncated to certain length and was replaceable, but could not be deleted in the ribozyme.
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1. Introduction

Ribonuclease P (RNase P) is one of the ubiquitous tRNA generating enzymes that take part in the 5′-maturation of the tRNA precursor. This enzyme commonly consists of one RNA subunit plus one or more protein subunits [1,2]. The RNA subunit acts as a major catalytic component in bacteria and some archaea, and provides the architecture of enzymes in eukarya [3]. Phylogenetic studies have revealed that there are several local domains in the RNA subunit of RNase P and the presence of many domains is commonly conserved beyond species [4]. Of these, the P4 domain and the L15/16 loop domain of bacterial enzymes have been the target of numerous studies: the P4 domain takes part in key magnesium ion binding [5,6] and the L15/16 domain takes part in the binding of CCA-3′ of the pre-tRNA substrate [7,8]. Many important bases are highly conserved in these domains. The J3/4 domain, located between the P3 and P4 domains, is also a conserved domain and takes part in magnesium ion binding [5,6]: replacement of bases in this domain drastically reduces the catalytic efficiency of the enzyme [9]. The tertiary structural model of RNA subunit of the *Escherichia coli* ribozyme suggests that the position and orientation of the above metal binding bases on J3/4 domain are supported and fixed by neighboring P2 and P3 domains [10,11]. Compared to the P2 and P4 domains, the P3 domain varies in sequence and length. In this paper, we prepared several P3 domain variants of *E. coli* RNase P and examined the effects of mutations in this enzyme.

2. Materials and methods

2.1. Preparation of RNAs and other materials

The *E. coli* RNase P RNA and the *E. coli* pre-tRNA$_3^{Pro}$ were prepared by in vitro transcription with *Thermo* T7 RNA polymerase (Toyobo) from pGEM-3Z-derived plasmids [12–14]. Every RNase P RNA contains an additional 5′-GGAATTC-3′ tag sequence (containing *Eco*RI site) at the 5′-end and an additional 5′-AAGCT-3′ tag sequence (derived from the *Hin*dIII site) at the 3′-end. The mutant RNase P RNAs were constructed using the restriction sites *Eco*RI (G^{-6}–C^{-1}) and *Ban*II (G^{72}–C^{77}) with synthetic DNAs that have a mutated sequence. The pre-tRNA was labeled at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase as described in the literature [12]. The *E. coli* RNase P protein was purified from *E. coli* BL21(DE3) cells harvesting a pET3a-derived plasmid as described in the literature [15]. Reconstitution of *E. coli* RNase P holoenzyme was done according to the method described in the literature [14,15]. The concentrations of RNase P RNA and pre-tRNA were measured spectrophotometrically by absorbance at 260 nm, and the concentration of the RNase P protein was measured with a BCA kit (Pierce). Other enzymes and chemicals were purchased from the commercial sources described in the literature [12].

2.2. RNase P reactions of pre-tRNA

The standard reaction mixture contained 50 mM Tris–HCl (pH 8.0), 100 mM NH$_4$Cl, 2.5–40 mM MgCl$_2$, 5% (w/v) polyethylene glycol 6000, 0.36 μM *E. coli* RNase P RNA, and 93.9 nM 5′-labeled pre-tRNA$_3^{Pro}$ in a total volume of 20 μl reaction mixture. The ribozyme reactions were done at 2.5, 5, 10, 20, or 40 mM magnesium ion concentrations. The holoenzyme reactions were done at magnesium ion concentrations of 1 or 2 mM. 5′-end labeled pre-tRNA$_3^{Pro}$ was used as a substrate for every reaction. *E. coli* RNase P protein was added to the reaction mixture so as to be equimolar with the RNase P RNA. The RNase P holoenzyme was reconstituted through incubation at 70 °C for 10 min and 37 °C for 1 h. The mixture was incubated at 37 °C for 1 h and the products were separated by 10% polyacrylamide gel electrophoresis [12]. Quantitative analyses of the reactions were made by measuring the photostimulated luminescence of the product bands using BAS-1800 (Fujifilm).

3. Results and discussion

3.1. RNase P reactions by P3 variants

We prepared four stepwise truncation mutants, denoted as Δ1, Δ2, Δ3, and Δ4, and one replaced mutant, denoted as TAR, of the P3 domain of *E. coli* RNase P RNA, and used

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them to examine the RNase P reactions of a pre-tRNA substrate. $\Delta 1$ has 31-nt length as the P3 domain (lacking G^{33}–C^{38} and G^{43}–A^{47}), $\Delta 2$ has 22-nt (lacking C^{29}–C^{38} and G^{43}–G^{52}), $\Delta 3$ has 8-nt (lacking G^{22}–C^{38} and G^{43}–G^{59}), and $\Delta 4$ has only 1-nt as P3 (lacking C^{21}–G^{61} regions of the wild-type RNA), respectively. TAR has 29-nt HIV-1 TAR RNA partial sequence instead of the P3 C^{20}–G^{61} region (see Fig. 1B; [16]). First, we examined the ribozyme reactions of these enzymes and second, the holoenzyme reactions. Fig. 2A shows the results of the ribozyme reactions of a 5′-end labeled pre-tRNA substrate at various magnesium ion concentrations. The results showed that the substrate pre-tRNA was cleaved at the same site by

every ribozyme. These results indicate that mutation of the P3 domain did not affect the cleavage site of the substrate by the ribozyme. The same results were obtained for the reconstituted holoenzyme (Fig. 3A).

On the other hand, the cleavage efficiency varied depending on the magnesium ion concentrations when the P3 domain was mutated. At 2.5 mM magnesium ion concentration, cleavage of the substrate was observed for only wild-type and $\Delta 2$ ribozymes; other ribozymes required higher concentrations of magnesium ion (Fig. 2B). At the above 10 mM magnesium ion, the presence of magnesium ion fully permitted the ribozyme reactions to cleave the substrate, whereas cleavage by the $\Delta 4$
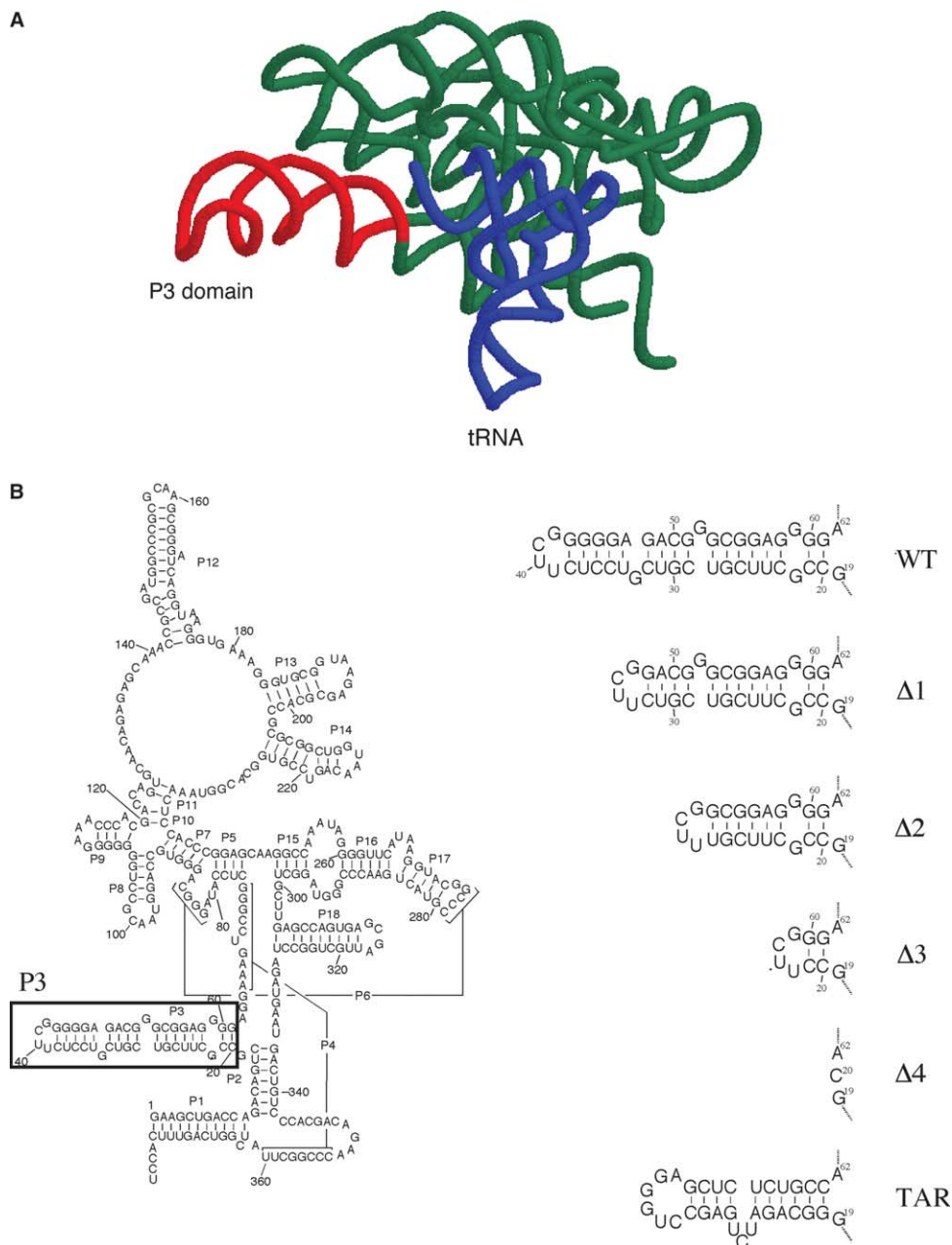

Fig. 1. (A) Illustrative representation of the *E. coli* RNase P ribozyme with a tRNA (*blue*). The P3 domain (*red*) is shown with other domains (*green*). The structure was generated from data by Chen et al. [11] using the graphics software 'RasMol for Macintosh' version 2.6. (B) The secondary structure of the *E. coli* RNase P RNA with derivatives of the P3 domain used in this study. The secondary structure of the *E. coli* RNA was from the RNase P website database [11].

ribozyme was not regained under these conditions. The presence of higher concentrations of magnesium ion also enhanced holoenzyme activity (Fig. 3B). Interestingly, the $\Delta 1$ and $\Delta 2$ variants demonstrated higher activities than the wild-type holoenzyme at 1 mM magnesium ion concentration. Also, the comparison of results for ribozyme and holoenzymes of the $\Delta 4$ variant showed that the presence of a protein component can recover enzymatic activity at 2 mM magnesium ion concentration that was not recovered at high concentrations of magnesium ion without the protein component.

The above results indicate that the P3 domain of *E. coli* RNase P RNA is able to be truncated to a certain length and is replaceable by other sequences. Mutation of this domain also affected the cleavage efficiency of a pre-tRNA substrate by the enzyme, but not drastically provided a certain length was allowed to remain.

3.2. Phylogenetic comparison

The phylogenetic comparative studies of Brown et al. [11] show that the RNA component of RNase P commonly contains a P3 domain that varies in length and in sequence. The length of the bacterial P3 domain ranges from 11 to 65 bases, while the archaeal tends to be shorter than those of bacteria, and the eukaryotic P3 domain tends to be longer (Fig. 4). Our results of stepwise truncation of the P3 domain showed that a certain length of this domain is required for efficient activity, at least in the case of the *E. coli* enzyme, but the same pattern was not observed with the archaeal enzymes. For example, the length of the P3 domain of the RNA subunit from *M. vannielli* and *M. thermolithotrophicus* has 9 bases; the former RNA has ribozyme activity but the latter RNA has no ribozyme activity [17]. And, in many cases, the length of the P3 domain does not seem to be related to the ribozyme activity of archaeal RNase P RNA; this pattern suggests that the length of the P3 domain does not directly determine the degree of the ribozyme activity of the RNA subunit.

What, then, is the role of the P3 domain? We cannot explain it now, but can only make assumptions. The tertiary structural model shows that the important metal binding site J3/4 domain is structurally supported by P2 and P4 helices and also the P3 domain (Fig. 1A). We assume that the P3 domain contributes to the correct location and orientation of
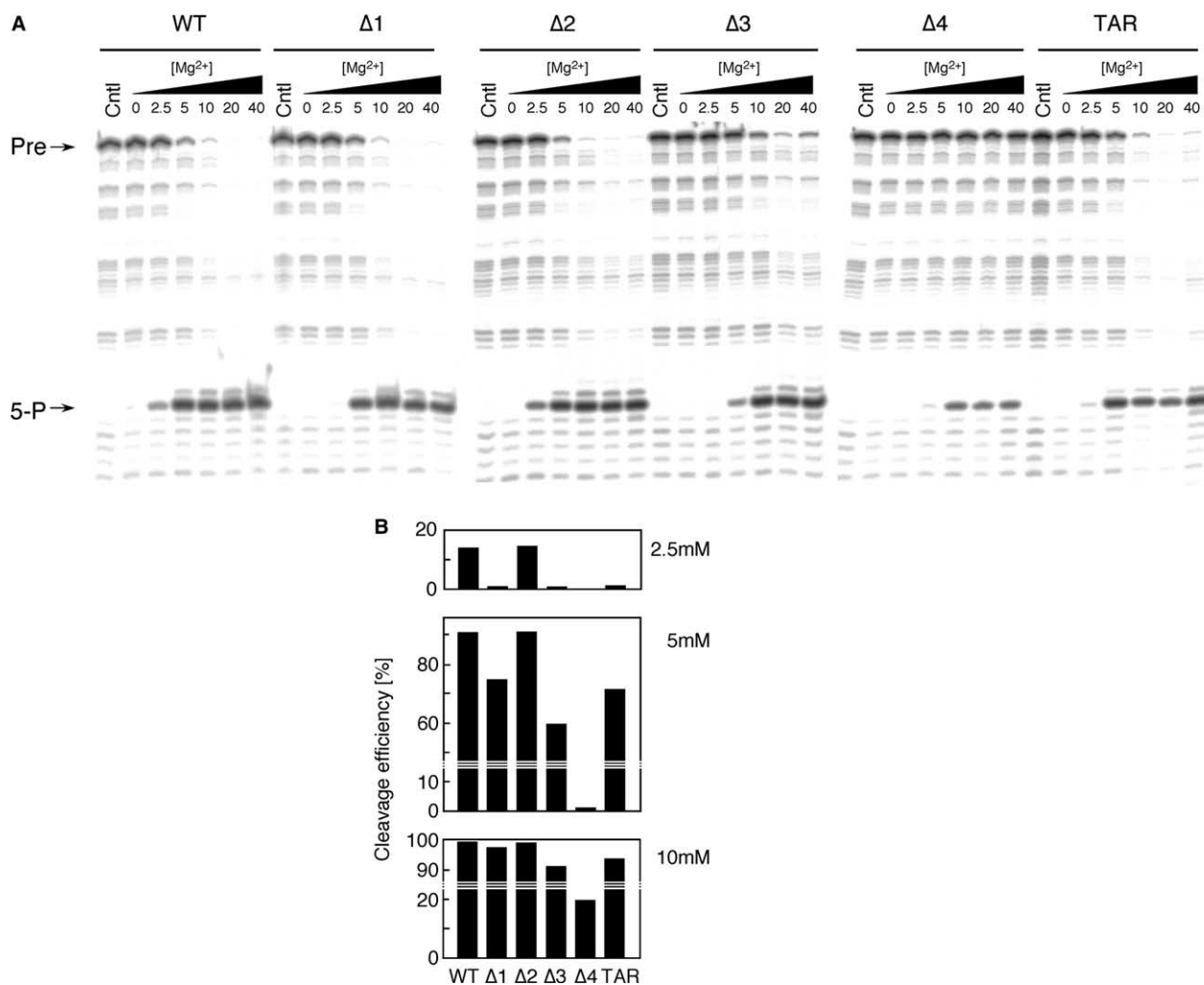

Fig. 2. Cleavage of pre-tRNA by RNase P ribozymes. (A) The ribozyme reactions were carried out at magnesium ion concentrations of 0, 2.5, 5, 10, 20, and 40 mM (93.9 nM *E. coli* pre-tRNA$_3^{Pro}$, 0.36 µM each of *E. coli* RNase P RNA variants, 50 mM Tris–HCl, 100 mM NH$_4$Cl, and 5% [w/v] polyethyleneglycol-6000; pH 8.0; 37 °C; 60 min). 'Pre' and '5-P' represent the pre-tRNA and the 5'-leader product, respectively. 'Cntl' represents the control reaction in the absence of the enzyme at magnesium ion concentration of 40 mM. (B) Quantitative analyses of ribozyme reactions at magnesium ion concentrations of 2.5, 5, and 10 mM.
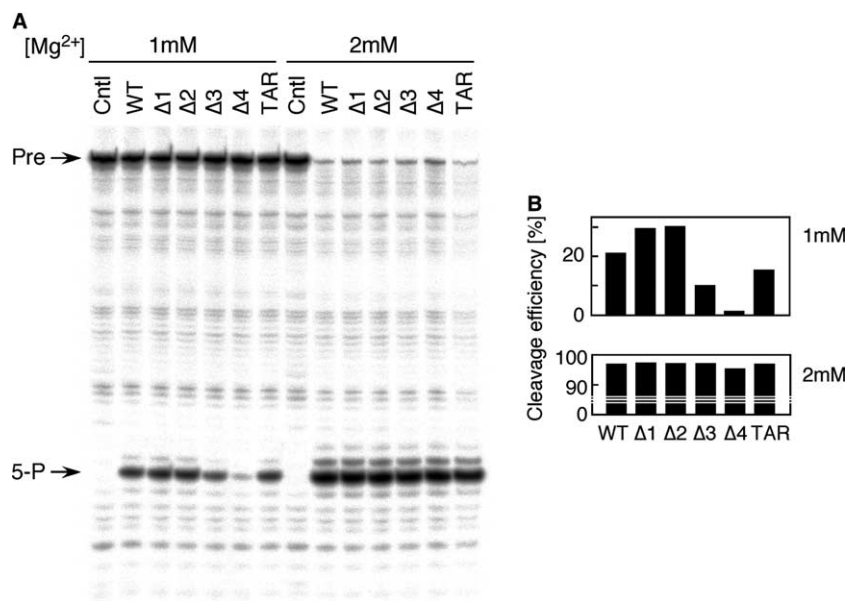
Fig. 3. RNase P reactions of pre-tRNA by holoenzymes. (A) The holoenzyme reactions were carried out at magnesium ion concentrations of 1 or 2 mM (93.9 nM *E. coli* pre-tRNA$_3^{Pro}$, 0.36 μM each of *E. coli* RNase P RNA variants, 0.36 μM *E. coli* RNase P protein, 50 mM Tris–HCl, 100 mM NH$_4$Cl, and 5% [w/v] polyethyleneglycol-6000; pH 8.0; 37 °C, 60 min). 'Pre' and '5-P' represent the pre-tRNA and the 5′-leader product, respectively, and 'Cntl' represents the control reaction in the absence of the enzyme at each magnesium ion concentration. (B) Quantitative analyses of holoenzyme reactions.
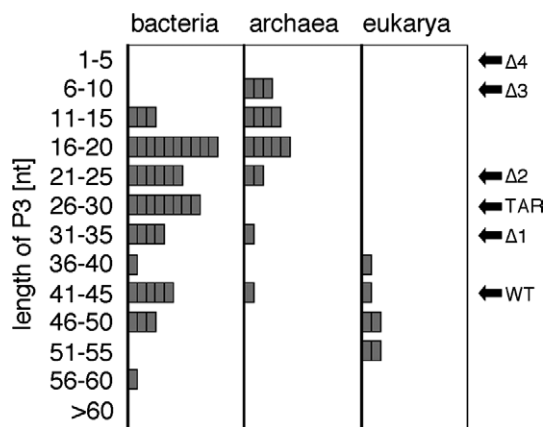

Fig. 4. Distribution of the length of the P3 domain of RNase P RNAs. 41 bacterial sequences and 16 archaeal sequences are shown. Six eukaryotic sequences are shown: *H. sapiens, G. gorilla, B. bufo, D. jeanneae, S. pombe,* and *S. cerevisiae.* The RNA sequences and the secondary structure predictions for them were from the RNase P database [11]. The lengths of the P3 derivatives for this study are also shown on the right.

the J3/4 metal binding domain, and therefore the P3 domain is structurally important for bacterial RNase P RNAs with respect to active center formation. The P3 domain may behave as a sticky tab that links the proximate ends of the P2 and J3/4 domains. This explanation is, of course, consistent with the crosslinking study of the protein component by Sharkady et al. [18]. Their results showed that the partial region of the P3 domain, proximate to the P2 and J3/4 domains, contacts the protein component. Comparison of ribozyme and holoenzyme results of the Δ4 enzyme of our study suggests that the protein component interacts with this region of the RNA component.

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