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RNase P RNA from *Prochlorococcus marinus*: contribution of substrate domains to recognition by a cyanobacterial ribozyme

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Abstract The molecular organisation of the *Prochlorococcus marinus rnpB* gene and the catalytic activity of the encoded RNA were characterised. Kinetic parameters for several pre-tRNA substrates were comparable to those from other eubacterial RNase P RNAs, although unusually high cation concentrations were required. The CCA-end of pre-tRNAs is essential for efficient turnover despite the lack of the canonical binding motif in *P. marinus* RNase P RNA. A *trnR* gene is located only 38 nt upstream the *rnpB* 5′ end on the complementary strand. This arrangement resembles those in the plastids of *Cyanophora* and *Porphyra* but not in any other bacterium.

Key words: RNase P; Ribozyme; Pre-tRNA processing; $tRNA^{Glu}$; Cyanobacterium; *Prochlorococcus marinus*

1. Introduction

RNase P, the ubiquitous enzyme required for 5′ end maturation of tRNAs, consists of essential RNA and protein subunits in all prokaryotes and most eukaryotic cell organelles. The RNA components from bacteria are catalytically active as ribozymes in vitro, whereas those from eukaryotes and archaea are not (reviewed in [1,2]). In contrast, the composition of RNase P from plastids seems extremely variable: an RNA component might be lacking entirely in green (i.e. Chl-*a*+*b*-containing) chloroplasts [3], and no significant homologies to known *rnpB* sequences can be identified in the genomes of these organelles, or a diatom plastid [4–6]. However, the photosynthetic organelles (cyanelles) of the eukaryotic unicellular alga *Cyanophora paradoxa* contain an *rnpB* gene for the RNA subunit of a ribonucleoprotein type RNase P [7,8]; a corresponding gene has also been identified on a red algal plastome [9]. Based on molecular phylogeny, the cyanelle of *C. paradoxa* has been positioned as an early branching group close to the base of all plastids, which are rooted within the cyanobacteria [10–12]. However, it is unknown which particular class within this large and very diverse systematic group might have given rise to the plastid lineage. To date, several complete [13–16] or partial [17] cyanobacterial *rnpB* gene sequences have been described. Only for one cyanobacterium, *Synechocystis* 6803, sequences adjacent to *rnpB* are known as a result of a whole genome analysis [18]. Only little information is available concerning the enzymatic activity or substrate recognition by these cyanobacterial RNA enzymes. So far, no data at all exist on the RNase P of any marine cyanobacteria, although a considerable diversity within this group can be supposed.

A typical representative of some marine cyanobacteria is *Prochlorococcus marinus* CCMP 1375. This abundant marine prokaryote was initially characterized as 'prochlorophyte' due to its pigment composition [19]. However, *P. marinus* and various relatives form a cluster on an own branch within the cyanobacterial radiation and thus should be considered as a group of atypical cyanobacteria [12,20]. The phylogenetic origin of *Prochlorococcus* has not been fully resolved; its very ancient descent, close to the basis of oxygenic photosynthesis, has been suggested [21]. In contrast, according to rRNA sequence data, it is phylogenetically closest to the evolutionarily younger marine cyanobacteria of the *Synechococcus* group A [20]. These reasons make *Prochlorococcus* an interesting model to study the molecular evolution of RNase P among the cyanobacterial radiation and beyond. Especially intriguing is the question to which of the structural groups the *P. marinus* RNase P RNA most closely resembles, and whether it is catalytically active like the bacterial type RNAs, or inactive like that from the cyanelle.

Of particular interest is the contribution of the pre-tRNA 3′-end towards substrate recognition by RNase P RNA. A pronounced influence of the CCA-terminus on product formation has been found for the A- and B-type RNase P ribozymes (e.g. [22,23]). In contrast to *E. coli*, tRNA genes from cyanobacteria and plastids do not encode the terminal CCA sequence [6,18,24,25]. In accordance, a motif corresponding to the well-conserved CCA-binding site in most other RNase P RNAs [26–29] is only found in a small subset of the known cyanobacterial sequences [13–17]. A unique feature of cyanobacteria and plastids is the presence of an unusually structured T-stem in $tRNA^{Glu}$, the tRNA required for porphyrin biosynthesis [30,31]. A participation of the T-stem and loop domain in substrate binding to the RNase P ribozyme has been established for *E. coli* and *B. subtilis* [32–35], but nothing is known about the mode of substrate recognition by the cyanobacterial RNAs.

In the present study, we have cloned and sequenced the *P. marinus* rnpB region. We have determined the optimal conditions for efficient catalysis by this ribozyme, and the kinetic parameters for different pre-tRNA substrates. In addition, we present the first analysis of substrate recognition by a cyanobacterial-type RNase P RNA lacking the CCA binding site.

2. Materials and methods

2.1. DNA isolation, cloning, and sequencing procedures

All recombinant DNA manipulations were performed as described [36] using pUC19 or pBluescribe M13 as vector and DH5-α or JM109 as host bacteria. Genomic DNA was islated from *P. marinus* CCMP 1375 according to [37]. The construction of a genomic cosmid library

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of *P. marinus* (SuperCos, Stratagene) will be described elsewhere (Hess et al., in preparation); a 2.4-kbp *Eco*RI fragment of the positive primary cosmid clone was subcloned into pUC19 to yield pPmarE2.4. The Sequenase 2.0 kit (United States Biochemical) was used together with universal forward and reverse primers and specific oligonucleotides (see below) to obtain the complete sequence of both strands. The University of Wisconsin GCG program package (Version 8) was used for data analysis.

2.2. Oligonucleotides

Oligonucleotides were either obtained from Eurogentec, or synthesized on a Gene-Assembler Plus (Pharmacia) and purified prior to use. Sequences in lower case letters indicate restriction sites introduced for cloning purposes. PmarGSP2: GTTACCCAGCAAGTTGG; PmarGSP3: ATAGATGATCACCCACTAACC; PmarGSP4: CCAACAGCCTCGAGCGGC; PmarF5′: CCCACGACCTACGGTTTAGG; PmarF3′: GAATGCAGGTTACTTTGCGTC; PmarET7: gcgaattcTAATACGACTCACTATAGGGAAAGCAGGAGAGGTCATC; Pmar3′B: gcggatccgacgctttcgAAAGAAAGCAGGAAGTAAGC; Glu5′: cgaattcGTATATGGCCCTCGTCTAGTGATGCCCCTATCGTCTAGTGG; GluCCA: gcctgcagGATGTCCGG; GluA73: gcggatccggatgAATACTCCCTACCCCCAGGGGAAG.

2.3. Polymerase chain reaction and nucleic acid hybridization

10 ng *P. marinus* CCMP 1375 genomic DNA was amplified (40 cycles: 1 min at 94°C, 30 s at 61°C, 1 min at 72°C) with primers cprp3′ and cprp5′ and cloned as described [7]. A hybridization probe was prepared by random labelling of the insert with the rediprime kit (Amersham). For Southern analysis of genomic DNA and library screening, membranes (GeneScreen Plus, DuPont; or HyBond N^+, Amersham) were hybridized in 7% SDS, 250 mM NaP_i, pH 7.2, at 58–60°C.

2.4. Test for catalytic activity

PmarET7 and Pmar3′B were used to amplify *rnpB* from pPmarE2.4 and to introduce a T7 promoter at the 5′ end, and appropriate restriction sites to allow efficient runoff transcription. Transcripts were obtained from *Bam*HI restricted pT7PmarRP and assayed for activity as described [7]. All ribozyme buffers contained 50 mM Tris-Cl, pH 8; monovalent cation concentration was optimized for pre-$tRNA^{Tyr}$ [38] at 250 mM $MgCl_2$ and the Mg^{2+}-dependence of the RNase P RNA reaction was determined in 3 M NH_4Cl.

Pre-$tRNA^{Glu}$ variants were prepared from *Fok*I cleaved templates containing the corresponding region of barley chloroplast DNA, which had been amplified with primers Glu5′ and GluCCA or GluA73. The ribozyme RNA was carefully renatured in assay buffer (50 mM Tris-Cl, pH 8, 3 M NH_4Cl, 250 mM $MgCl_2$) immediately before use. Steady state kinetic parameters at 25 nM RNase P RNA were determined from several independent experiments.

3. Results

3.1. The genomic region encoding RNase P RNA from Prochlorococcus marinus

A cosmid clone hybridizing to the PCR amplified probe was isolated and a 2.4 kbp *Eco*RI fragment was subcloned for sequence analysis of ca. 1000 base pairs containing *rnpB* and adjacent genes (Fig. 1). Co-linearity between the cosmid and the genome was verified by Southern blots (data not shown). The 5′ end of *P. marinus* RNase P RNA, as determined by primer extension, is located at guanosine 254 and uridine 253 (data not shown). The 3′ end of the RNA, as deduced from the secondary structure and mapping experiments, is located at position 640, followed by two short stretches of T residues and a continuous reading frame starting 48 nt downstream of *rnpB*. Several inverted repeats (IRs) are located within the sequenced region: between positions 3–107, and 672–685. A gene for $tRNA^{Arg}_{CCU}$ (*trnR*) is present in opposite orientation upstream of *rnpB*, between positions 215 and 142 (Fig. 1). In agreement with tRNA genes from cyanobacteria and plastids, but different from those of *E. coli*, the 3′CCA-sequence is not included in this gene.

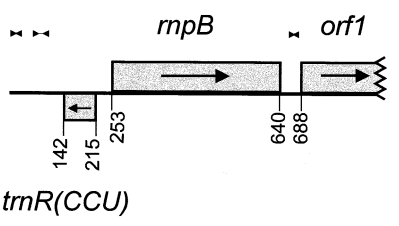

Fig. 1. Schematic representation of the *rnpB* region from *Prochlorococcus marinus*. The position and orientation of the RNase P RNA gene (*rnpB*), the downstream ORF1, and the $tRNA^{Arg}_{CCU}$ gene (*trnR*) encoded on the opposite strand are indicated by shaded boxes and arrows therein. Inverted repeats (IRs) are represented by pairs of inversely oriented black triangles in the upper part of the figure. Numbers refer to the start and endpoints of genes as used in the text.

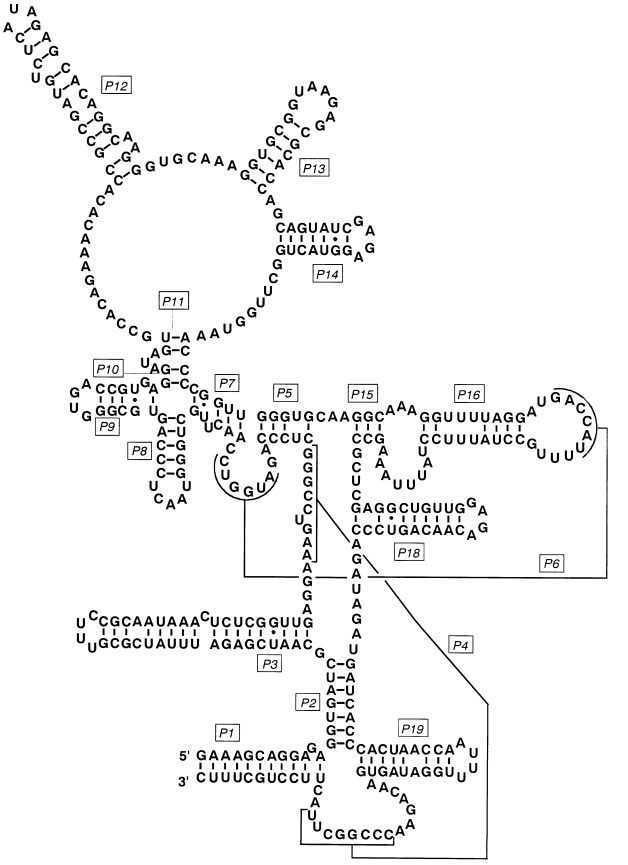

Fig. 2. Secondary structure of *P. marinus* RNse P RNA modelled after the bacterial RNase P RNA consensus structure. Designation of ribozyme domains is according to [33].

3.2. Structure of the P. marinus RNase P RNA

The postulated RNA secondary structure (Fig. 2) clearly indicates that the *P. marinus* RNA belongs to the cyanobacterial type [13–17,39]. The long-distance base pairing P6 [40] may potentially be extended to 6 base pairs in *P. marinus*, as for the other cyanobacterial-type RNAs including those from the *C. paradoxa* and *Porphyra* plastids [7,39]. An important structural feature shared with RNase P RNAs from most cyanobacteria (except *Pseudoanabaena*, *Oscillatoria*, and *Pro-*

chlorothrix) and the two plastids is the extended asymmetrical loop joining the stems P15 and P16. The corresponding domain in *E. coli* contains the 5′-GGU-3′ motif, which is involved in binding of the substrate CCA end [26–29]. However, the homologous loop in *P. marinus* RNase P RNA, as in most cyanobacterial and the two plastid RNAs, does not contain this sequence. The presence of three consecutive U-U 'mismatches' in helix P16 of *P. marinus* RNase P RNA is unique within the cyanobacterial group; a similar situation is only found in the cyanelle RNA, where a single U-U is found in this domain [7]. In contrast, all other cyanobacterial RNase P RNAs possess a 2–4-base bulge within an otherwise fully paired P16 [13–17,39]. A peculiarity of the *P. marinus* RNA is an unpaired U-U in helix P5; a mismatch at this position has to date been described only in two bacterial isolates assigned to the α-purple subgroup [41].

3.3. Influence of ionic conditions and pre-tRNA structure on RNase P RNA activity

RNase P RNA from *P. marinus* requires high concentrations of both Mg^{2+} and monovalent cations: at 250 mM $MgCl_2$, optimal substrate turnover is only observed above 2 M NH_4Cl (Fig. 3A). At 3 M NH_4Cl, a direct dependence of product formation on [Mg^{2+}] is observed below 150 mM, with no significant further increase above this concentration (Fig. 3B). The steady state parameters K_m and k_{cat} for cleavage of the different substrates under optimum conditions are given in Table 1.

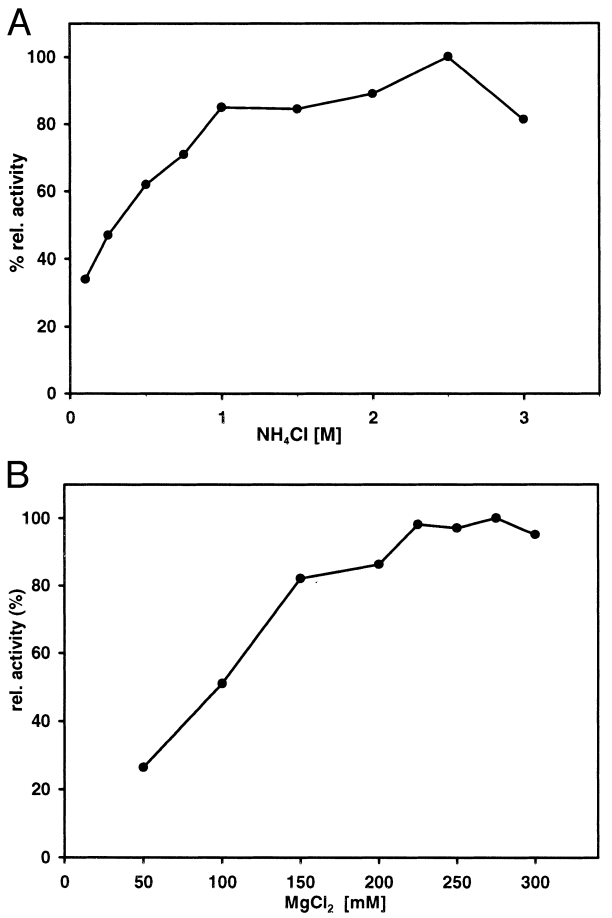

Fig. 3. A: Dependence of *P. marinus* RNase P RNA activity on NH_4^+ concentration, determined at 250 mM Mg^{2+}. B: Mg^{2+} requirement of the *P. marinus* RNase P RNA reaction, determined at 3 M NH_4Cl.

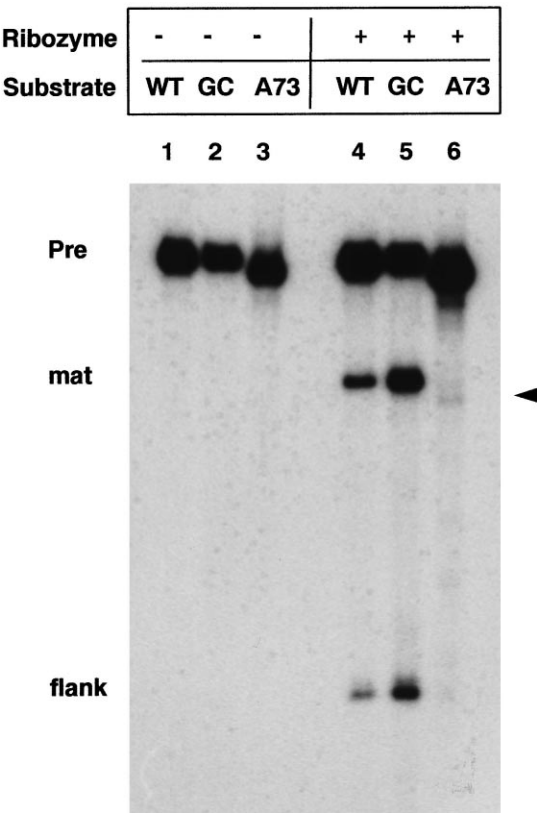

Fig. 4. Influence of the T-stem sequence and the presence of the CCA end on pre-$tRNA^{Glu}$ cleavage by *P. marinus* RNase P RNA. Presence or absence of ribozyme, and also the type of substrate used (wild-type pre-$tRNA^{Glu}$, the G53-C61 mutant, or the deletion mutant lacking the CCA terminus) are indicated above each lane. Substrate and ribozyme concentration were 20 nM and 10 nM, respectively; reactions were run at 37°C for 30 min. The position of the faint band corresponding to the 5′ mature product lacking CCA is indicated on the right side of the panel by a small arrowhead.

The contributions of conserved tRNA domains to recognition by the ribozyme were analysed using pre-tRNA variants which differ in the conserved T-domain, or in their 3′ terminus. For wild type pre-$tRNA^{Glu}$ containing the unique A53–U61 base pair, product formation is slightly reduced compared to the variant with the GC pair which is conserved in all other tRNAs (Fig. 4; Table 1). In contrast, the presence of the 3′ CCA sequence is crucial for efficient pre-tRNA recognition: product formation is barely detectable for the deletion mutant ΔCCA. The kinetic parameters for cleavage of this substrate have not been determined due to the low amount of product formation.

Table 1
Steady state kinetic parameters for cleavage of different pre-tRNAs by the *P. marinus* RNase P RNA ribozyme

	K_M (μM)	k_{cat} (min^{-1})
E. coli pre-$tRNA^{Tyr}$	0.57 (0.13)	0.58 (0.15)
Chloroplast pre-$tRNA^{Glu}$ (wt)	1.34 (0.05)	0.077 (0.002)
Chloroplast pre-$tRNA^{Glu}$ (GC)	1.20 (0.19)	0.103 (0.009)

4. Discussion

4.1. Organisation of the rnpB region

A single tRNA gene is located in close proximity and in head-to-head-orientation to *rnpB* in *P. marinus* (Fig. 1). Thus, the gene arrangement in this prokaryote is more similar to that found in the *Cyanophora* and *Porphyra* plastids [7–9] than to other cyanobacteria described to date. This organization may resemble an ancient arrangement (partially) conserved in the two plastid genomes, in *Prochlorococcus*, and possibly also in other cyanobacteria. Alternatively it might simply reflect the selection towards genome minimization in the organelles and in *P. marinus* CCMP 1375, the latter having a genome about half the size of other free-living cyanobacteria (1.8–2.0 Mbp; Hess, unpublished). No 'eubacterial-type' promoter sequences are found immediately upstream of the postulated transcription initiation sites of the *Prochlorococcus* RNA genes. However, the very short distance of only 38 bp between the first nucleotides of *rnpB* and of *trnR*, respectively, suggests that the two promoters may be overlapping each other, as described previously for the cyanelle of *C. paradoxa* [24]. Transcription termination of *rnpB* likely occurs within the short stretches of thymidines downstream of the structural gene, a motif frequently described near the end of cyanobacterial genes and thought to function as transcription terminator [14]. As expected for a non-heterocyst forming member of the cyanobacterial radiation, no short tandemly repeated repetitive (STRR) elements [14,17] are present within or near *rnpB* in *P. marinus*.

4.2. Structural and functional properties of P. marinus RNase P RNA

If compared to other cyanobacterial RNase P RNAs, *P. marinus* RNA shows several structural peculiarities which may lead to local destabilization of helices P5 and P16 in the ribozyme core. Interestingly, all these regions consist of single or consecutive U-U 'mismatches', which are comparable in stability to A-C if they are embedded within a helical context [43,44]. These minor deviations from the optimal RNA structure may be alleviated by the high concentrations of mono- and divalent cations required for optimal function of this ribozyme. A similarly strong dependence of cleavage activity on high ionic strength has previously been described for mutant RNase P RNAs lacking a structural domain which may exert a stabilizing effect on the overall structure [45]. The RNAs from the cyanobacterium *P. hollandica*, and from *B. subtilis* which belongs to the structurally different 'B-type', require similar salt conditions [16,46]. The *P. marinus* RNase P RNA (Fig. 2) belongs to a different structural class than the *P. hollandica* RNA [16]. This is an interesting observation, because there are several common physiological properties (e.g. in pigmentation and the composition of the photosynthetic apparatus [42]) in these two organisms which differentiate them from other cyanobacteria.

The kinetic parameters of *P. marinus* RNase P RNA for the cleavage of bacterial and chloroplast-derived substrates (Table 1) are well within the range described for other bacterial RNase P RNAs, specifically those from the two structurally different cyanobacteria *Synechocystis* 6803 and *Prochlorothrix* [14,16,17]. An adaptation of RNase P to the respective natural substrates in each organism can be expected, hence the determination of substrate requirements of the cyanobacterial enzyme is a reasonable approach to reveal possible differences in function between this and other, more thoroughly studied types of RNase P RNAs. Cyanobacteria, like plastids, contain a single $tRNA^{Glu}$ species which differs from the tRNA consensus by the unique A53–U61 base pair at the distal end of the T stem. Analysis of T-stem variants showed only small differences in their kinetic behaviour (Fig. 4, Table 1): as found previously for the *Prochlorothrix* ribozyme, the G-C mutant gives a slightly better product yield than the wild type containing the A-U pair. This observation is in agreement with the higher k_{cat} of the G-C mutant. The slightly lower K_M of this substrate, corresponding to a higher affinity, is more difficult to understand in this context.

The *P. marinus* ribozyme does not contain the GGU-sequence which has been identified as CCA-binding motif in other bacteria [26–29], but is missing in most cyanobacterial RNase P RNAs. Thus, the behaviour of the *P. marinus* RNA towards substrates differing in their 3′ end is of particular interest: it might be expected that the CCA terminus, which is not encoded in tRNA genes in these organisms ([18,25]; this study), is less stringently required for substrate recognition. Surprisingly, this is not the case at all: product formation is barely detectable for the ΔCCA variant. This is strikingly similar to the results obtained for the only other cyanobacterial RNase P analysed in this respect [16]. Thus, it will be interesting to investigate in the future how the *P. marinus* ribozyme – and the majority of cyanobacterial RNase P RNAs which lack the canonical CCA binding motif – recognize their substrate and orient it into the active site so that correct cleavage can occur. Further, our data show the need for additional studies to understand the possible relevance of overlapping promoters of *rnpB* genes and of tRNA genes located immediately upstream to *rnpB* in various organisms.

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