Sequence and functional characterization of RNase P RNA from the chl *a/b* containing cyanobacterium *Prochlorothrix hollandica*

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Abstract Only a few complete sequences and very limited functional data are available for the catalytic RNA component of cyanobacterial RNase P. The RNase P RNA from the chl *a/b* containing cyanobacterium *Prochlorothrix hollandica* belongs to a rarely found structural subtype with an extended P15/16 domain. We have established conditions for optimal in vitro ribozyme activity, and determined the kinetic parameters for cleavage of pre-tRNA^{Tyr}. Analysis of pre-tRNA mutants revealed that the T-stem sequence only plays a modulating role, whereas the CCA end is essential for efficient product formation.

Key words: RNase P; Ribozyme; Pre-tRNA processing; tRNA^{Glu}; Cyanobacterium; *Prochlorothrix hollandica*

1. Introduction

RNase P, the ubiquitous enzyme required for 5′ end maturation of tRNAs, contains an essential RNA subunit in prokaryotes, eukaryotic nuclei, mitochondria of fungi and a primitive protist, and plastids of red algae and Glaucophytes; the bacterial RNA component is catalytically active as a ribozyme in vitro [1–6]. In contrast, the enzyme composition in most mitochondria, and in chloroplasts of higher plants and most algae, remains enigmatic: an RNA component cannot be identified in enzyme preparations, and is not encoded on the respective organelle genomes [3,7–10]. The close phylogenetic relationship between cyanobacteria and plastids renders them suitable tools for studies on the molecular evolution of RNase P, although it is not clear at present which of the diverse cyanobacterial groups might be closest to the still unidentified ancestor of the plastid lineage (reviewed in [11]). A small number of cyanobacteria with a pigment composition similar to green chloroplasts had formerly been grouped together as 'Prochlorophytes'. Based solely on this similarity, they were tentatively considered the closest relatives of these organelles. Molecular phylogeny data, however, revealed that they do not form a natural group, but belong to different branches within the cyanobacterial radiation [11]. To date, only a few complete ([12–14]; Hess, Fingerhut and Schön, unpublished) and several partial [15] cyanobacterial RNase P RNA sequences have been described. Their proposed secondary structures fall into two related subtypes, defined by the presence or absence of an extended ('branched') P15/16 domain. A phenomenon unique to cyanobacteria is the apparent absence of a CCA binding site in the majority of RNase P RNAs, whereas this 5′-GGU-3′ motif is highly conserved in other bacteria [16]. In agreement with this peculiarity, and in contrast to most eubacteria, tRNA genes from cyanobacteria and plastids do not encode the 3′ terminal CCA sequence ([17,18]; Hess et al., unpublished). Another unique feature of cyanobacteria and plastids is the presence of an unusually structured T-stem in tRNA^{Glu}, the tRNA required for porphyrin biosynthesis [19,20]. The participation of the 3′ end and T-stem in substrate binding to the RNase P ribozyme has been established for RNase P RNA from other structural classes [21–27]. Thus, it is of particular interest to analyze the contribution of these domains to substrate recognition by the cyanobacterial-type ribozymes.

Given the structural variability even within the small number of known cyanobacterial RNase P RNAs, it is important to include members of divergent branches in a study aimed at understanding the evolution of RNase P RNA within this extensive radiation and into the plastid lineage. We have chosen, as a representative of the diverse group of chl *a+b* containing cyanobacteria, the freshwater species *Prochlorothrix hollandica* [28] and determined its complete RNase P RNA sequence. Optimal reaction conditions and kinetic parameters have been determined for pre-tRNA^{Tyr}. The contributions of two conserved substrate domains, T-stem and 3′ end, towards recognition by this ribozyme have been analyzed. This is the first report of a mutant substrate analysis for a cyanobacterial RNase P RNA.

2. Materials and methods

2.1. Cell growth and nucleic acid isolation

P. hollandica PCC 9006 was obtained from the Pasteur collection and grown in medium BG 11 with nitrate reduced to 2 mM [29] at 20°C under a 14 h light cycle; cells were harvested by centrifugation and quick-frozen in liquid N_2. For preparation of genomic DNA, thawed cells were dispersed with a syringe and adjusted to 20 mM Tris-Cl pH 8.0, 10 mM Na_2EDTA. Lysozyme (0.1 mg/ml final concentration) and RNase A (10 ng/ml) were added and the suspension was incubated on ice for 15 min. After supplementation with SDS (1%) and proteinase K (50 μg/ml), the incubation was continued for 30 min at 37°C. DNA was then isolated by extraction with TE-saturated phenol and chloroform-isoamyl alcohol (24:1). Further purification of the DNA phase was achieved by chromatography on Nucleobond AX100 columns (Macherey-Nagel) as described by the manufacturer; genomic DNA was eluted with buffer N5 and concentrated by isopropanol precipitation. For total RNA preparations, RNase and proteinase treatments were omitted from this purification scheme.

2.2. Cloning and sequencing procedures

All recombinant DNA manipulations were performed as described [30]. Universal forward and reverse primers and specific oligonucleotides (see below) were employed together with the Sequenase 2.0 kit (United States Biochemical) to obtain the complete sequence of both strands. Data analysis was performed with the programs FASTA or BESTFIT of the University of Wisconsin GCG program package (Version 8).

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2.3. Oligonucleotides

Oligonucleotides were either synthesized on a Gene-Assembler Plus (Pharmacia) and purified on Nucleobond AX5 columns (Macherey-Nagel), or obtained from Eurogentec and used directly. Sequences in lower-case letters indicate restriction sites introduced for cloning purposes. Pho-GSP2: CACGCGCACTGGGCGTTAC; Pho-GSP3: GCGGTACACCGCAGCGCC; Pho-GSP5: CGAGAGGTGCTGGCTCGG; T7Pho5′: gcgcTAATACGACTCACTATAGGGTGGCGGAGAAGTAG; Pho3′Bst: gcgcttcgaaAACGAAGAAGTTGGAC.

2.4. Polymerase chain reactions

10 ng *P. hollandica* genomic DNA was amplified with the primers cprp3′ and cprp5′ (corresponding to the conserved region P4) and cloned as described [5] to give pPhoRP. The 5′ end of RNase P RNA was determined by RACE from cDNA primed with cprp3′; two successive rounds of amplification were performed with an anchor primer (provided with the Gibco-BRL RACE kit) and GSP3 or GSP2, respectively. For 3′ RACE, total RNA was first polyadenylated (yeast poly(A) polymerase, USB); cDNA was then primed and amplified with a 3′ anchor primer (Boehringer Mannheim RACE kit) and cprp5′ or GSP5. The T7 promoter and a *Bst*BI restriction site for efficient runoff transcription were introduced by amplification of the *rnpB* coding region with T7Pho5′ and Pho3′Bst; the blunt ended product was cloned into the *Sma*I site of pUC19 to give pT7PhoRP. To exclude the introduction of mutations, several independent clones were sequenced for all PCR products.

2.5. Test for catalytic activity

RNase P RNA transcripts were obtained from *Bst*BI-restricted template and assayed for activity as described [5] at a ribozyme concentration of 6.2 nM. Ribozyme reaction buffers were composed of 50 mM Tris-Cl pH 8.0 and the following salts: 2 M NH_4Cl, 250 mM $MgCl_2$ (P1); 3 M NH_4Cl, 250 mM $MgCl_2$ (P2); or 3 M NH_4Cl, 25 mM $MgCl_2$ (P3). Cleavage reactions were performed at 37°C, under the conditions detailed in the figures. Steady-state kinetic parameters for *Escherichia coli* pre-tRNA^{Tyr} [31] were obtained in P2 at substrate concentrations between 25 and 1600 nM. Results were quantitated with a PhosphorImager (Molecular Dynamics).

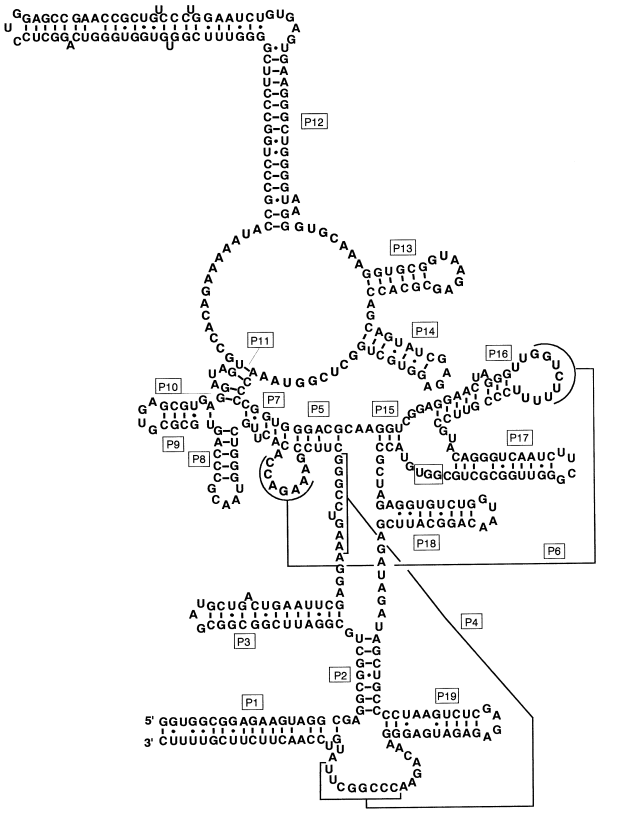

Fig. 1. Secondary structure of *P. hollandica* RNase P RNA modelled after the bacterial RNase P RNA consensus structure. Ribozyme domains are designated according to [26]; the GGU motif involved in tRNA binding is boxed.

3. Results and discussion

3.1. Structure of the P. hollandica RNase P RNA

P. hollandica RNase P RNA fully conforms to the bacterial consensus and can be folded into a cyanobacterial-type secondary structure, including the postulated tertiary interactions between P8 and the tetraloops L14 and L18, respectively (Fig. 1; [13–15,32]). Due to the extremely long P12 helix typical of many cyanobacteria, and the rarely found branched P15/16/17 domain, *P. hollandica* RNase P RNA is one of the largest of this group. This unusual structure is shared only with *Pseudanabaena* and *Oscillatoria* among the cyanobacteria. As in the latter, and different from the majority of cyanobacterial RNAs, the GGU motif required for binding of the pre-tRNA CCA end is present in the *P. hollandica* RNA ([21–24], boxed in Fig. 1). Unlike the situation in green sulfur bacteria and *Planctomyces*, but similar to *Chlamydia*, the distal end of P16 provides one strand of the long-distance interaction P6 [16,33]. As observed for the other cyanobacterial-type RNAs including those from the *Chlamydia paradoxa* and *Porphyra* plastids [4,5,16], helix P6 may potentially be extended to six base pairs in *P. hollandica*. No short tandemly repeated repetitive (STRR) elements [13,15] are present within this RNase P RNA, as expected for a non-heterocyst-forming member of the cyanobacterial radiation. Remarkably, the *P. hollandica* ribozyme contains a relatively high number of non-Watson-Crick base pairs, which may even occur in clusters, like in the central part of P12. Specifically, a C-U mismatch is present in P5, at the position of a U-U in *P. marinus* (Hess et al., unpublished). In contrast to U-U, which does not exert a destabilizing role when embedded within a helix, C-U can only form an isomorphous pair when it is protonated [34,35]. The only other examples for a similar P5 structure in bacterial RNase P RNA are restricted to two members of the α-purple group [16,32].

3.2. Catalytic activity of P. hollandica RNase P RNA

The *P. hollandica* ribozyme prefers high concentrations of both Mg^{2+} and monovalent cations: optimal substrate turnover is observed at 250 mM $MgCl_2$ and 3 M NH_4Cl (buffer P2). Cleavage products can barely be detected at 25 mM $MgCl_2$ (P3; see Fig. 2); the faint additional bands visible under these conditions are possibly due to some non-specific degradation, caused by misfolding of the substrate. This requirement for high mono- and divalent cation concentrations is unlike the optimum reaction conditions described for *E. coli* M1 RNA [36], but rather resembles those required by the RNase P RNAs from *Bacillus subtilis*, which belongs to the structurally different B-type [37,38]; by mutant RNAs lacking P12, which may exert a stabilizing effect on the overall structure [39]; or by the *P. marinus* RNA (Hess et al., unpublished), which resembles the *P. hollandica* ribozyme in possessing several non-canonical base pairs in conserved helical regions. Thus, it can be concluded that a high ionic strength environment may help to alleviate minor deviations from an optimal RNase P RNA structure by supporting the correct folding of the ribozyme core.

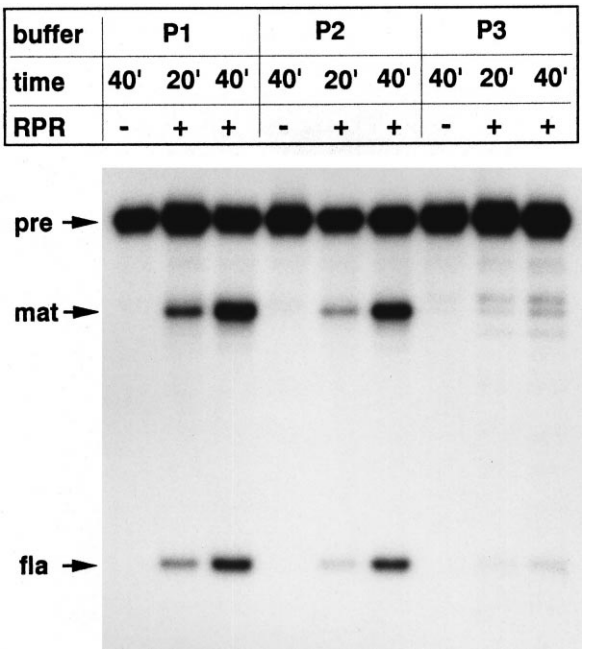

Fig. 2. Activity of *P. hollandica* RNase P RNA under different ionic conditions. Cleavage of pre-tRNA^{Tyr} was assayed in the buffers described in Section 2. Reactions were terminated at the times indicated above each lane. The positions of precursor, mature tRNA and 5′ flank are given on the left of the panel.

3.3. *Influence of pre-tRNA structure on recognition by the P. hollandica ribozyme*

The steady-state parameters K_m and k_{cat} for cleavage of *E. coli* pre-tRNA^{Tyr} are 1.7 μM and 6.4 min^{-1}, respectively. This is well within the range described for other bacterial RNase P RNAs like those from *E. coli* [40,41], *T. thermophilus* [42], *B. subtilis* [43], or the cyanobacterium *Synechocystis* 6803 [13,15].

Because an adaptation of RNase P to specific substrates in each organism can be expected, the determination of structural requirements of the cyanobacterial enzyme might reveal differences in function between this and more thoroughly studied RNase P RNAs like those from *E. coli* or *B. subtilis*. 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 [19,20]; another feature distinct from most bacteria is the lack of an encoded 3′ CCA terminus in their tRNA genes [17]. We have investigated the influence of these tRNA domains on recognition by the *P. hollandica* ribozyme by employing pre-tRNA mutants which differ in the conserved T-domain, or in their 3′ terminus (Fig. 3A). Analysis of the T-stem variants revealed only small differences (Fig. 3B): the wild type containing the A-U pair gives a slightly lower product yield than the G-C mutant, which resembles the majority of naturally occurring tRNAs. Since substrate and product bind with about equal affinities to RNase P RNA [44], the observed difference may only be explained by a time delay in the correct positioning of the wild type substrate. In the *E. coli* and *B. subtilis* ribozymes, the T-stem domain of pre-tRNA is situated near the P7-P11 cruciform in the E-S complex [26,45]. Possibly, the A-U pair in the pre-tRNA^{Glu} substrate does not match perfectly to the docking site on the *P. hollandica* ribozyme. The corresponding region differs in several details between the cyanobacterial type and the other structural classes of RNase P RNAs [27]; thus, it may be more flexible and possibly allow an adaptation to such unusual substrates.

In contrast to the minor influence of the T-stem sequence, the presence of the 3′ CCA end is crucial for efficient pre-tRNA cleavage: product formation is barely detectable for the deletion mutant (Fig. 3B). This behavior towards substrates lacking the 3′ terminus is not unexpected, because the *P. hollandica* ribozyme belongs to the small subgroup of cyanobacterial RNase P RNAs containing the 'GGU motif', which has been identified as a CCA binding site in other bacteria [21–24]. Although it is not encoded in tRNA genes

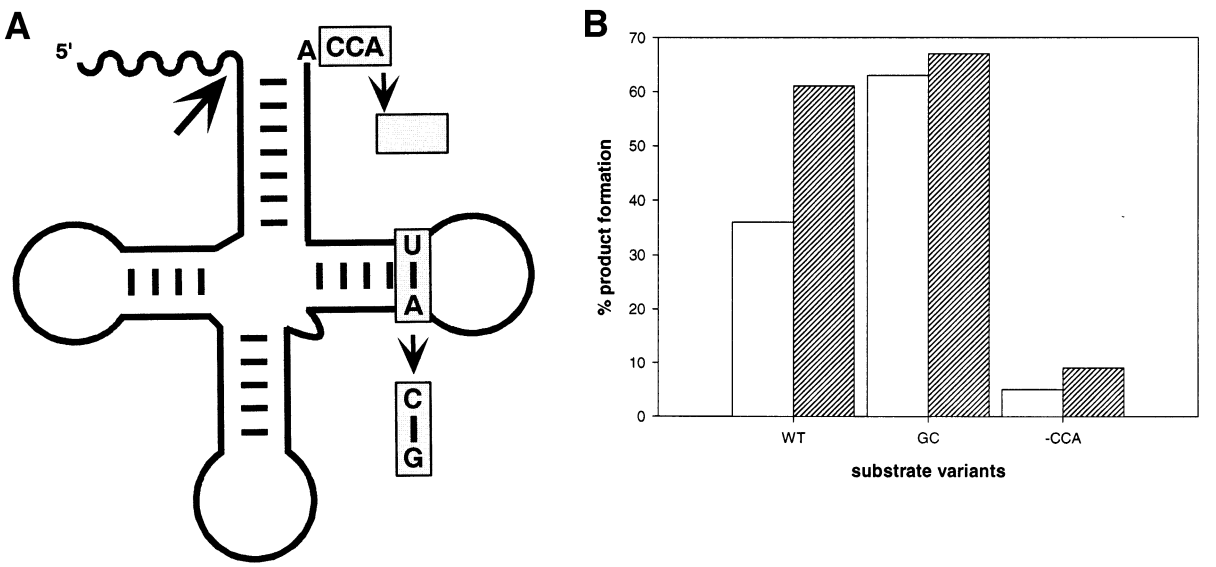

Fig. 3. A: Schematic representation of pre-tRNA^{Glu} mutants. The deletion of the 3′ terminal CCA and the changes in the T-stem are indicated by boxes. The 5′ flank is drawn as a wavy line and the position of the phosphodiester bond cleaved in the RNase P reaction is shown by a large arrowhead. B: Influence of substrate structure on pre-tRNA cleavage by *P. hollandica* RNase P RNA. Cleavage of wild type pre-tRNA^{Glu} (WT; A53-U61), the G53-C61 mutant (GC), or the CCA deletion mutant (−CCA) was measured after 30 min (white bars) or 60 min (hatched bars).

in cyanobacteria [17], the CCA terminus is obviously essential for efficient product formation by the *P. hollandica* RNA. This result is in striking contrast to a report concerning the *Synechocystis* 6803 RNase P RNA: there, even an inhibition of cleavage by the presence of the CCA end was observed [15]. It is not clear at present whether this divergent behavior is due to the different structure of the P16/17 domain in these two cyanobacterial ribozymes.

4. Conclusions

Our analysis of substrate recognition by *P. hollandica* RNase P RNA clearly showed that product formation by a cyanobacterial RNase P RNA is only slightly influenced by the T-stem sequence, but is critically dependent on the presence of the 3′ terminal CCA end. To better understand the function of these ribozymes, it will be important to see whether the cyanobacterial RNAs conform to the same mode of substrate binding as those from *E. coli* and *B. subtilis*, with the T-stem docking near the P7-P11 cruciform structure. Also, it is of interest to know whether in vivo the CCA terminus is needed for efficient pre-tRNA cleavage, or whether the protein component alleviates this requirement, as has been shown for other RNase P RNA types [23]. The solution of these questions will be the scope of our future work on *P. hollandica* RNase P RNA.

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