# Functional reconstitution of RNase P activity from a plastid RNA subunit and a cyanobacterial protein subunit

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Received 19 November 1998

**Abstract The plastid (cyanelle) from the Glaucocystophyceae alga *Cyanophora paradoxa* contains an RNase P RNA subunit (P RNA) similar to the cyanobacterial P RNA. We have synthesized this RNA by in vitro transcription and analyzed its activity in the absence or presence of the RNase P protein subunit (P protein) from *Escherichia coli* and the cyanobacterium *Synechocystis* sp. PCC 6803. In contrast to the bacterial P RNA, the cyanelle P RNA is not active in the absence of protein in any of the conditions tested. A functional enzyme could be reconstituted with the *Synechocystis* protein but not with the *E. coli* protein. This is the first demonstration of RNase P activity reconstitution from organellar and bacterial subunits.**



*Key words:* Plastid evolution; RNA catalysis; Ribonuclease P; Cyanobacteria; *Cyanophora* cyanelle

## 1. Introduction

Ribonuclease P (RNase P) is a ubiquitous endoribonuclease that generates the mature 5′ ends of tRNAs through a single endonucleolytic cleavage of pre-tRNAs [1]. In Bacteria, Archaea, Eucarya and mitochondria, the enzyme is a ribonucleoprotein containing an essential RNA subunit. In Bacteria, the RNA subunit (P RNA) is catalytically active in vitro, in the absence of the protein subunit (P protein), under high magnesium and high ionic strength conditions [2]. In chloroplasts of higher plants, there is biochemical evidence that the enzyme is composed solely of protein [3,4]. No gene encoding a homologue to the bacterial P RNA has been identified in the several fully sequenced chloroplast genomes, except in the genome of the chloroplast from the red alga *Porphyra purpurea* [5] and in the cyanobacterium-like plastid (cyanelle) from the protist *Cyanophora paradoxa* [6,7]. The RNase P activity from cyanelles is sensitive to micrococcal nuclease, and the cyanelle P RNA has been shown to copurify with RNase P activity, suggesting that this RNA is an essential component of the cyanelle RNase P [7]. Although cyanelle P RNA is predicted to have a secondary structure similar to the bacterial homologue, it differs at certain positions from the consensus structure [7]. In addition, it has a high AT content. Cyanelle P RNA is not active in vitro without protein in any of the conditions tested ([7]; this work). This has prompted the suggestion that the cyanelle RNase P enzyme represents an evolutionarily intermediate form between the bacterial RNA based enzyme and the plant chloroplast protein based enzyme [7].

To date, no information is available on the protein composition of the cyanelle RNase P. Moreover, the cyanelle genome does not encode a protein homologous to bacterial P proteins [8].

To study if the cyanelle P RNA is functionally equivalent to its bacterial counterpart, we have analyzed the enzymatic activity of in vitro transcribed cyanelle P RNA in the absence or presence of the purified P protein from *Escherichia coli* and from the cyanobacterium *Synechocystis* sp. PCC 6803. We have found that activity could be reconstituted from cyanelle P RNA and *Synechocystis* P protein but no activity could be reconstituted with *E. coli* P protein in any of the conditions assayed. In addition, we have modified by site directed mutagenesis the unusual UCAA sequence of loop L14 in the cyanelle P RNA to the conserved GNRA tetraloop found in Bacteria, resulting in a completely inactive RNA.

## 2. Materials and methods

### 2.1. Preparation of P RNAs

P RNAs were prepared by in vitro transcription with T7 RNA polymerase from template plasmids as described [9]. The cyanelle *rnpB* gene (encoding P RNA) was amplified by PCR from purified cyanelle DNA from *C. paradoxa* Pringsheim strain LB555, generously provided by Dr. Wolfgang Löffelhardt (Vienna University). The forward primer (5′-CCCGGAATTCTAATACGACTCACTATAG-AAACGAATTTAATTAATG-3′) contained an *Eco*RI site, the T7 RNA polymerase promoter, and overlaps the 5′ end of the coding sequence. The reverse primer (5′-CCCAAGCTTTAAACGAACT-TAATTTTAAGC-3′) contains a *Hin*dIII site and a *Dra*I site overlapping the 3′ end of the coding sequence. To facilitate transcription by T7 RNA polymerase, a G was added at the 5′ end of the *rnpB* gene. A PCR fragment of the expected size was obtained, purified, digested with *Eco*RI and *Hin*dIII and ligated into pUC19 that had been treated with the same enzymes. The plasmid obtained, after digestion with *Dra*I, was used for in vitro run-off transcription with T7 RNA polymerase [9]. The RNAs obtained would contain the same ends and sequence as those expected for the authentic cyanelle P RNA [10] except for the extra G at the 5′ end.

Template plasmids for in vitro transcription of *E. coli* P RNA (M1 RNA) [9] and *Synechocystis* P RNA [11] have already been described.

### 2.2. Purification of proteins

P protein from *E. coli* (C5 protein) was purified from cultures of BL21(DE3) carrying plasmid pARE7 as described [9]. P protein from *Synechocystis* sp. PCC6803 was purified from cultures of BL21(DE3) carrying plasmid pARA2 as described [12].

### 2.3. RNase P activity assays

A pre-tRNA$^{Tyr}$ from *E. coli* [13] or pre-tRNA$^{Gln}$ from *Synechocystis* [12] were used as substrates. Pre-tRNAs were prepared by in vitro run-off transcription with T7 RNA polymerase. After transcription the RNAs were dephosphorylated with calf intestinal phosphatase and labeled at the 5′ end with polynucleotide kinase and [$\gamma$-$^{32}$P]ATP. RNase P holoenzyme was reconstituted by direct mixing of the in

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*Abbreviations:* P RNA, RNA subunit of RNase P; P protein, protein subunit of RNase P; pre-tRNA, precursor of tRNA


PII: S0014-5793(98)01621-4

vitro transcribed P RNA with a 5–10-fold molar excess of the purified P protein and incubation for 5 min at 37°C immediately before the assays. Assays were done as described [9] by incubating at 37°C an aliquot of the reconstituted holoenzyme with $^{32}$P-labeled substrate in assay buffer. Reaction products were analyzed by electrophoresis on 20% acrylamide/7 M urea gels and autoradiography.

*2.4. Site directed mutagenesis*

Mutations U184G and A186G were introduced simultaneously in the cyanelle *rnpB* gene by site directed mutagenesis with the oligonucleotide 5′-GCAAACCAGTTAGATTGCGAAATCTATACTTAGTAAACC-3′. The U.S.E. Mutagenesis Kit from Pharmacia was used for this purpose.

## 3. Results

It has been previously found that in vitro transcribed cyanelle P RNA is not catalytically active on several pre-tRNAs under a variety of conditions [7]. The pre-tRNAs used were an *E. coli* pre-tRNA$^{Tyr}$ and two barley chloroplast pre-tRNAs. We have confirmed this negative result with an additional pre-tRNA substrate (pre-tRNA$^{Gln}$) from the cyanobacterium *Synechocystis* sp. PCC 6803 under a variety of conditions, included high magnesium concentration and high ionic strength. Under none of the conditions tested could we observe RNase P activity (not shown). Therefore, the cyanelle RNase P seems to be strictly dependent on protein for its activity. Analysis of the primary structure of cyanelle P RNA shows several positions that are invariant in Bacteria but are different in cyanelle P RNA [7]. When the predicted secondary structure of cyanelle P RNA is analyzed, some of the proposed tertiary interactions present in bacterial P RNAs are missing (Fig. 1). This could explain the inability of cyanelle P RNA to fold into a functional conformation in the absence of protein and therefore the lack of activity. Since the nature of the protein subunit(s) of the cyanelle RNase P is unknown, we decided to analyze if a bacterial P protein could substitute for the cyanelle P protein(s) in restoring catalytic activity of cyanelle P RNA. We analyzed the reconstitution with the P proteins from *E. coli* (C5 protein) and *Synechocystis* sp. PCC6803 and assayed the activity with two substrates, pre-tRNA$^{Gln}$ from *Synechocystis* and pre-tRNA$^{Tyr}$ from *E. coli*. A functional RNase P holoenzyme could be reconstituted with cyanelle P RNA and *Synechocystis* P protein (Fig. 2, lane 9) but not with cyanelle P RNA and *E. coli* P protein (Fig. 2, lane 8). Our preparations of the P proteins were fully functional because both of them could reconstitute RNase P activity with the *Synechocystis* P RNA (Fig. 2, lanes 6 and 7), as previously described [12]. None of the protein or RNA subunits used had any background detectable activity by themselves at the concentrations and assay buffer used for holoenzyme reconstitution (Fig. 2, lanes 1–5). *Synechocystis* and *E. coli* P RNAs are both active in the absence of protein but only under high magnesium concentration and high ionic strength conditions [2,12], very different from the conditions used here for holoenzyme reconstitution. Therefore, the activity observed in Fig. 2, lanes 6, 7, and 9 must be due to the reconstituted holoenzyme. Similar qualitative results were obtained with two different substrates from two organisms, pre-tRNA$^{Gln}$ from *Synechocystis* and pre-tRNA$^{Tyr}$ from *E. coli* (compare panels A and B in Fig. 2). Therefore, it can be concluded that a functional RNase P holoenzyme can be reconstituted with cyanelle P RNA and the cyanobacterial P protein but not with the *E. coli* P protein. The assay conditions used were those

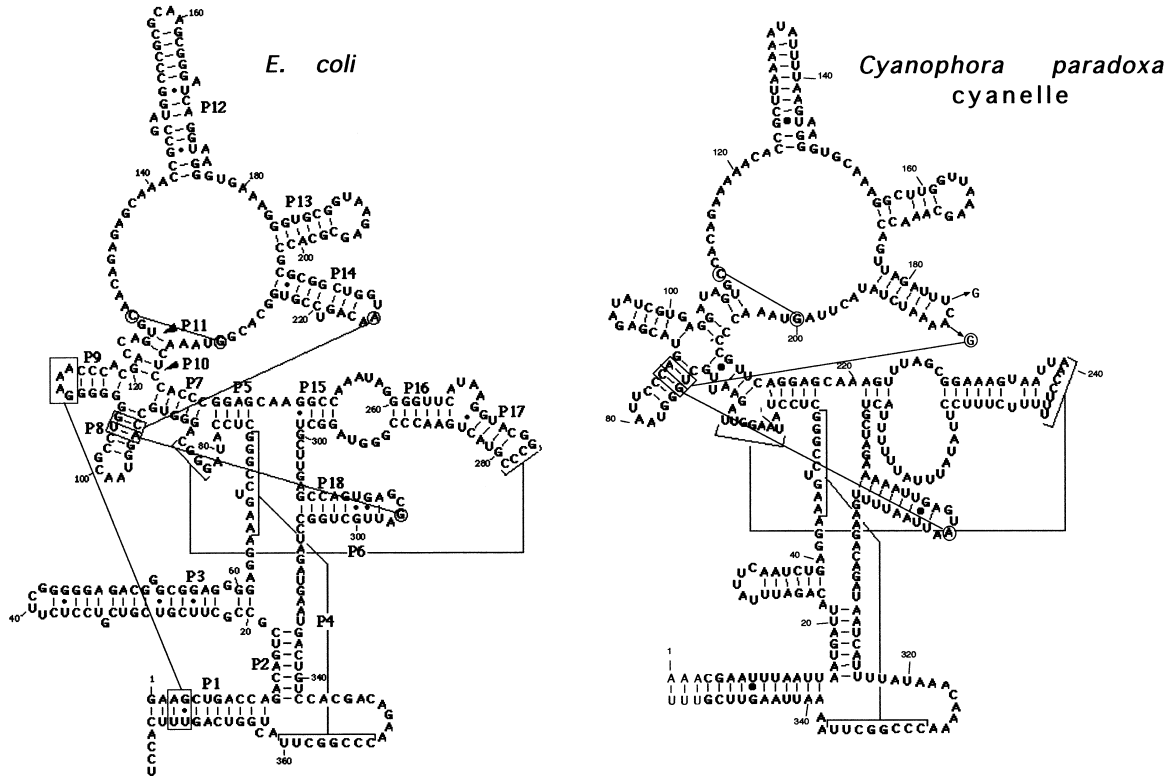


Fig. 1. Secondary structure models of *E. coli* and *C. paradoxa* cyanelle P RNAs. The structures shown are adapted from the RNase P Database [18]. Tertiary interactions and pseudoknots are shown by lines connecting the interacting regions. In the cyanelle structure the nucleotides substituted in the loop L14 to generate mutant U184G/A186G are also shown.

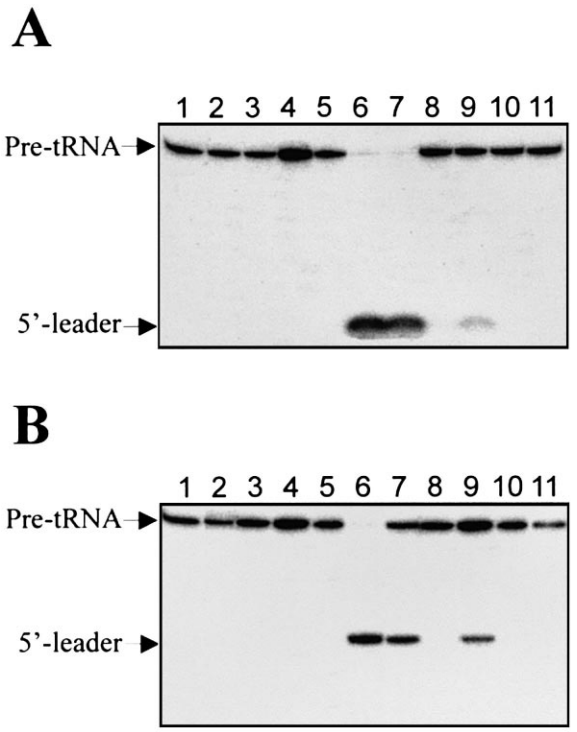


Fig. 2. RNase P activity on *Synechocystis* pre-tRNA$^{Gln}$ (A) or *E. coli* pre-tRNA$^{Tyr}$ (B) of holoenzyme reconstituted with subunits of different origins. 1, *E. coli* P protein (1 μM); 2, *Synechocystis* P protein (1 μM); 3, *Synechocystis* P RNA (5 nM); 4, cyanelle P RNA (200 nM); 5, cyanelle P RNA(U184G/A186G) (200 nM); 6, *Synechocystis* P RNA (5 nM) and *E. coli* P protein (50 nM); 7, *Synechocystis* P RNA (5 nM) and P protein (50 nM); 8, cyanelle P RNA (200 nM) and *E. coli* P protein (1 μM); 9, cyanelle P RNA (200 nM) and *Synechocystis* P protein (1 μM); 10, cyanelle P RNA(U184G/A186G) (200 nM) and *E. coli* P protein (1 μM); 11, cyanelle P RNA(U184G/A186G) (200 nM) and *Synechocystis* P protein (1 μM). The P RNAs, P proteins, or reconstituted RNase P holoenzymes were incubated with $^{32}$P-labeled pre-tRNA$^{Gln}$ (A) or pre-tRNA$^{Tyr}$ (B) for 2 h at 37°C in 50 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$. Reactions products were separated on a 20% polyacrylamide/urea gel and detected by autoradiography.

found optimal for the *Synechocystis* RNase P holoenzyme (50 mM $MgCl_2$, absence of monovalent cations) (Pascual and Vioque, unpublished), which differ from the optimal assay conditions generally used in assaying bacterial RNase P. In the standard assay conditions for the *E. coli* holoenzyme (10 mM $MgCl_2$, 400 mM $NH_4Cl$), no activity could be detected with cyanelle P RNA reconstituted either with *Synechocystis* or *E. coli* P proteins (not shown).

The loop in helix P14 (L14) is in bacteria a GNRA tetraloop that has been proposed to interact with helix P8 (Fig. 1) on the basis of covariation of the sequences [14]. Three-dimensional models of bacterial P RNAs also take into account this interaction [15,16]. In the cyanelle P RNA, the sequence of L14 is not a GNRA tetraloop but is instead UCAA, a sequence which is never found in bacterial P RNAs. We have changed this sequence to GCGA in the mutant U184G/A186G. In this way, the tertiary interaction between L14 and P8 is theoretically restored according to the rules established for this interaction [14]. However, the mutant P RNA failed, like the wild type P RNA, to show activity by itself with any of the conditions tested (not shown). Furthermore, it also lacked detectable activity in reconstitution assays with either the *E. coli* P protein (Fig. 2, lane 10) or the *Synechocystis* P protein (Fig. 2, lane 11).

## 4. Discussion

The P RNA from the cyanelle has a primary and secondary structure similar to the P RNA from Bacteria, however, it lacks catalytic activity in the absence of protein. There are several differences in highly conserved nucleotides as well as tertiary interactions between cyanelle P RNA and bacterial P RNA that could account for the lack of activity of this RNA. The fact that higher plant chloroplasts seem to lack an RNA component in their RNase P and the postulated evolutionary proximity between cyanelles and cyanobacteria have raised the suggestion that the cyanelle RNase P represents a transition from the RNA based enzyme of bacteria to the protein based enzyme of higher plants chloroplasts [7]. Our success in reconstituting a functional RNase P with the cyanelle P RNA and a cyanobacterial P protein supports the idea that cyanelle P RNA is essentially similar in structure and function to the cyanobacterial P RNA. Protein and RNA subunits of bacterial RNase P are in general interchangeable. For instance, *E. coli* RNase P subunits can reconstitute a functional enzyme with the corresponding subunits from *Bacillus subtilis* [2] or *Synechocystis* [12]. However, our data indicate that the cyanelle P RNA can reconstitute activity with only the *Synechocystis* P protein but not with the *E. coli* P protein (compare lanes 9 and 10 in Fig. 2). These results support a closer structural and functional similarity of the cyanelle RNase P to the cyanobacterial enzyme than to the *E. coli* enzyme, as could be expected from the specific evolutionary relationship between cyanobacteria and plastids. For the same reason, a cyanelle P protein similar to the cyanobacterial P protein should exist. However, no homologue to the bacterial P protein could be detected in the fully sequenced cyanelle genome. Therefore, if this homologue indeed exists, it must be encoded in the nucleus, where most of the plastid genes have migrated [17]. Identification of this protein and its gene would require a better characterization of the cyanelle RNase P. So far, the only partial purification reported for the cyanelle RNase P did not provide information on the protein composition of the enzyme [7]. Antibodies raised against the *Synechocystis* P protein [12] do not recognize specifically any cyanelle protein (Pascual and Vioque, unpublished).

Another interesting point is the identification of the structural reasons why the cyanelle P RNA is not catalytically active in the absence of protein. Probably, the RNA cannot fold into a functional structure unless the protein is present. Biochemical analysis of the structure of the cyanelle P RNA should give some relevant information. We have analyzed the effect on catalytic activity of the modification of loop L14 in the cyanelle P RNA (UCAA) to the conserved structure of loop L14 in bacterial P RNAs (a GNRA tetraloop). The mutant RNA was not active by itself, as the wild type, and it had also lost the ability to reconstitute RNase P activity with the *Synechocystis* P protein. Interestingly, loop L14 has been modified in *E. coli* without detectable effect on the activity of the P RNA in vitro [14], which suggests that while in *E. coli* P RNA the tetraloop:helix tertiary interaction can be replaced without detrimental effects, in the cyanelle P RNA whatever interactions are established by the UCAA tetraloop are disrupted by its change to a GCGA tetraloop, abolishing

the ability to reconstitute a functional holoenzyme. This result points to a rather different organization of tertiary interactions in the cyanelle P RNA, perhaps involving the P protein and explaining the lack of catalytic activity of cyanelle P RNA by itself.

*Acknowledgements:* This work was supported by Grants from the Human Frontier Science Organization (RG291/1997), Dirección General de Enseñanza Superior (PB97-0732) and Junta de Andalucía (CVI-0215). A. Pascual was supported by a predoctoral fellowship from the Spanish Ministry of Education and Culture. We are grateful to Dr. Wolfgang Löffelhardt for the purified cyanelle DNA and to Dr. Jesús de la Cruz for critical reading.

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