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Conditional Expression of RNase P in the Cyanobacterium *Synechocystis* sp. PCC6803 Allows Detection of Precursor RNAs

INSIGHT IN THE *IN VIVO* MATURATION PATHWAY OF TRANSFER AND OTHER STABLE RNAs*

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We have constructed a strain (CT1) that expresses RNase P conditionally with the aim to analyze the *in vivo* tRNA processing pathway and the biological role that RNase P plays in *Synechocystis* 6803. In this strain, the *rnpB* gene, coding for the RNA subunit of RNase P, has been placed under the control of the *petJ* gene promoter (P_{petJ}), which is repressed by copper, cell growth, and accumulation of RNase P RNA is inhibited in CT1 after the addition of copper, indicating that the regulation by copper is maintained in the chimerical P_{petJ}-*rnpB* gene and that RNase P is essential for growth in *Synechocystis*. We have analyzed several RNAs by Northern blot and primer extension in CT1. Upon addition of copper to the culture medium, precursors of the mature tRNAs are detected. Furthermore, our results indicate that there is a preferred order in the action of RNase P when it processes a dimeric tRNA precursor. The precursors detected are 3′-processed, indicating that 3′ processing can occur before 5′ processing by RNase P. The size of the precursors suggests that the terminal CCA sequence is already present before RNase P processing. We have also analyzed other potential RNase P substrates, such as the precursors of tmRNA and 4.5 S RNA. In both cases, accumulation of larger than mature size RNAs is observed after transferring the cells to a copper-containing medium.

Ribonuclease P (RNase P)[1] is an ubiquitous enzyme responsible for generating the 5′ end of precursors of tRNAs (pre-tRNAs) by a single endonucleolytic cleavage (1, 2). In bacteria, the enzyme is composed of an RNA subunit and a protein subunit. Both subunits are essential *in vivo*. The RNA subunit is the catalytic component, and, under appropriate conditions *in vitro*, it can cleave substrates in the absence of the protein (3).

In addition to processing by RNase P at the 5′ end, pre-tRNAs are also processed at the 3′ end, where several nucleases are involved (4, 5). The precise order of events in the processing of pre-tRNAs in bacteria is not well known. It seems that 3′ processing precedes 5′ processing, but the maturation process, at least at the 3′ end, behaves as a random process with the participation of multiple redundant enzymatic activities (6). In cyanobacteria, the 3′-CCA end of tRNAs is not encoded in the genome and must be added post-transcriptionally. *In vitro*, cyanobacterial RNase P prefers substrates lacking the 3′-CCA (7), suggesting that RNase P cleavage might precede addition of CCA by tRNA nucleotidyltransferase, but at present there is no knowledge on the *in vivo* pathway.

Pre-tRNAs are not the only substrates of RNase P. Several other natural and artificial substrates have been identified. In *Escherichia coli*, RNase P processes RNAs such as the precursors to 4.5 S RNA (8), tmRNA (9), and a small phage RNA (10).

We describe in this paper the construction of a strain from *Synechocystis* that expresses conditionally the RNA component of RNase P. The analysis of this strain has shown that RNase P is essential in this cyanobacterium. In addition, we have determined the *in vivo* processing pathway of pre-tRNAs and established that other RNAs, such as 4.5 S RNA and tmRNA, are also dependent on RNase P for their processing.

EXPERIMENTAL PROCEDURES

Growth and Transformation of Synechocystis 6803—Synechocystis cells were grown photoautotrophically at 30 °C in BG11c medium (11) under continuous illumination conditions (50 microeinsteins of white light m^{-2} s^{-1}). For RNase P RNA depletion, cells were grown in BG11c medium with no $CuSO_4$ added. When the culture was at a density of 2–3 μg of chlorophyll/ml, 5 μM $CuSO_4$ was added to the medium and incubation continued for 48 h before RNA extraction. Chlorophyll concentration was determined as described previously (12).

DNA Manipulation—All manipulations were performed by standard methods (13) or as recommended by the manufacturers of the enzymes used. DNA was extracted from *Synechocystis* cells as described previously (14).

Plasmids—The plasmid pSCT1 was used to introduce a copy of the *rnpB* gene under the control of the *petJ* promoter (P_{petJ}) in the *glnN* locus of *Synechocystis*. To construct pSCT1, a kanamycin resistance cassette (15), followed by P_{petJ} and by the *rnpB* gene lacking its promoter (P_{petJ}-*rnpB*) was placed inside the *glnN* gene, which, in turn, was inserted within pBluescript. The P_{petJ} and *rnpB* DNA fragments were synthesized by PCR using oligonucleotides that contained convenient restriction sites for cloning. The sequences of both fragments were checked by sequencing. The P_{petJ} fragment extends from positions −472 to +3 of the *petJ* gene, relative to the start of translation. Therefore, it contains the transcriptional and translational regulatory sequences of *petJ*. The *rnpB* fragment extends from position 1 to 437, relative to the transcription start site of the gene. Since pSCT1 does not replicate in *Synechocystis*, after transformation and selection of kanamycin resistant cells, we expected to obtain transformants bearing the genomic structure indicated at the bottom of Fig. 1*A* in the *glnN* locus. Detailed information on the construction of the pSCT1 plasmid is available from the authors. This plasmid can be used to insert different protein coding genes in the *glnN* locus and to express them under the control of the *petJ* promoter. For that purpose, the desired gene should be flanked by a *Sph*I site containing the ATG start codon (in the case of a protein

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[1] The abbreviations used are: RNase P, ribonuclease P; pre-tRNA, precursors of tRNA; tmRNA, transfer messenger; PCR, polymerase chain reaction.

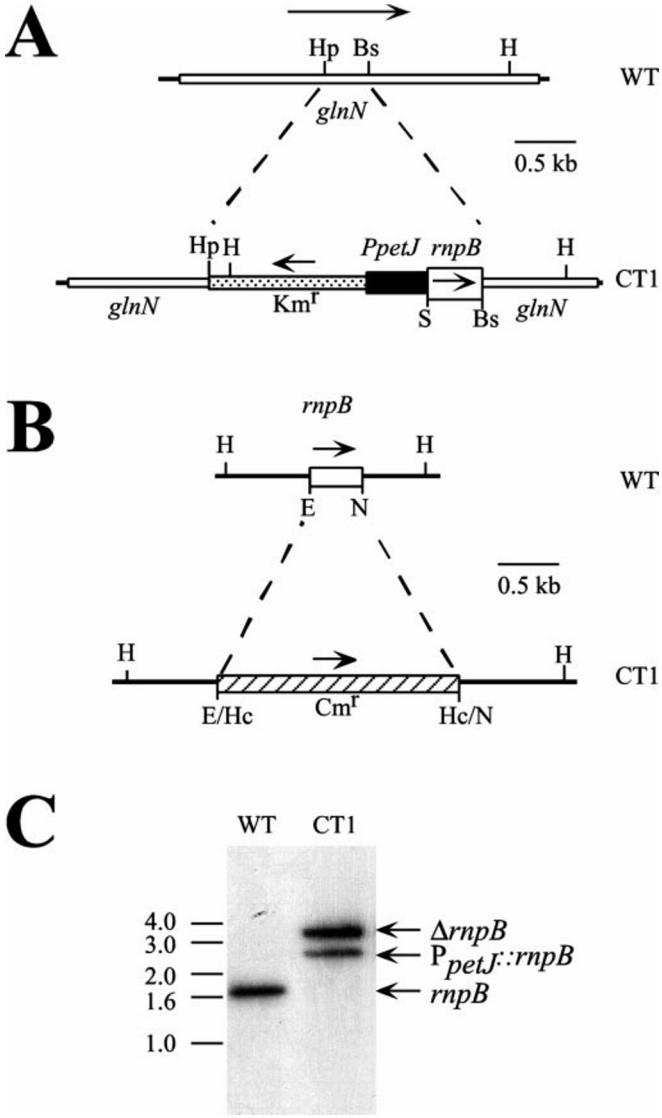

FIG. 1. **Genomic structure of CT1, a *Synechocystis* strain with conditional expression of the *rnpB* gene.** *A*, structure of the *glnN* region in the wild type *Synechocystis* strain and in the CT1 mutant. *Open rectangles* represent the *glnN* locus. *Dotted* and *black filled boxes* represent the kanamycin resistance cassette and the *petJ* promoter fragment, respectively. The *arrow-containing rectangle* represents the promoterless *rnpB* gene. *Arrows* indicate the direction of transcription. *B*, structure of the *rnpB* region in the wild type *Synechocystis* strain and in the CT1 mutant. The *open rectangle* represents the *rnpB* gene, whereas the *striped rectangle* represents the chloramphenicol resistance cassette. *C*, Southern blot analysis of genomic DNA from the wild type and CT1 strains. Genomic DNA was digested with *Hin*dIII and hybridized with the *Hin*dIII fragment containing the *rnpB* gene represented in the upper part of *B*. Fragment sizes are in kilobases. Restriction site abbreviations: *Hp*, *Hpa*I; *Bs*, *Bst*EII; *H*, *Hin*dIII; *N*, *Nco*I; *S*, *Sph*I; *E*, *Eco*NI; *Hc*, *Hin*cII.

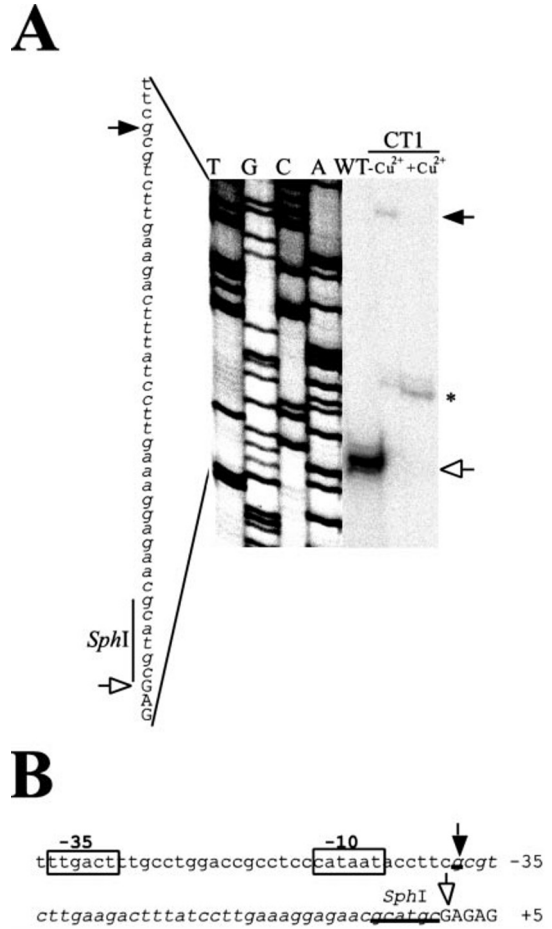

FIG. 2. **Repression by copper of *rnpB* expression in the CT1 strain.** *A*, total RNA from wild type cells incubated in the presence of Cu^{+2} and from CT1 strain grown in the absence or in the presence of 5 Cu^{2+} were annealed to the PTR3 oligonucleotide and extended with reverse transcriptase as described under "Experimental Procedures." A sequencing ladder generated with the same primer from plasmid pSCT1 is also shown. The *white arrow* indicates the transcription start point of the wild type *rnpB* gene. The *black arrow* indicates the transcription start point of the P_{petJ}*::rnpB* fusion. The *asterisk* indicates a band of unknown origin, whose nature is discussed in the text. *B*, sequence of the promoter region of the P_{petJ}*::rnpB* fusion. Nucleotides corresponding to the wild type RNase P RNA are shown as *uppercase*. Additional nucleotides present in the RNase P RNA transcribed from the *petJ::rnpB* fusion are shown in *italics*. The −35 and −10 sequences upstream of the transcription start point are *boxed*. The *Sph*I site used to construct the fusion is *underlined*. *Arrows* are the same as in *A*.

coding gene) and by a *Bst*EII site located after the stop codon. Both restriction sites are unique in pSCT1, where they flank the *rnpB* gene. Thus, a unique cloning step will be enough to replace the *rnpB* gene of pSCT1 by the desired gene. Since the *Sph*I site of pSCT1 contains the ATG start codon of the *petJ* coding region, the resulting construct will contain a perfect fusion of the desired protein, which will be translated using the ribosome binding site of *petJ*.

Plasmid pSCT2 was used to mutate the wild type *rnpB* allele present in the *Synechocystis* strain bearing the P_{petJ}*-rnpB* chimerical gene. It was constructed using plasmid pAV1000, which contains a 4.2-kilobase pair fragment of genomic DNA from *Synechocystis* 6803 carrying the *rnpB* gene (16). To construct pSCT2, pAV1000 was digested with *Eco*NI and *Nco*I and ligated with a chloramphenicol resistance cassette (15). This plasmid does not replicate in *Synechocystis*. Thus, after selection of chloramphenicol-resistant cells, we expected to obtain transformants bearing the genomic structure indicated at the bottom of Fig. 1*B* in the *rnpB* locus.

RNA Analysis—Total RNA was extracted from 25 ml of culture by vortexing cells in the presence of phenol-chloroform and acid-washed baked glass beads (0.25–0.3 mm in diameter; Braun, Melsungen, Germany) as described previously (17). For Northern blots, samples containing about 15 μg of total RNA were separated on a 8% polyacrylamide, 7 M urea gel, transferred to nylon membranes (Hybond N-plus, Amersham Pharmacia Biotech), and hybridized with ^{32}P-labeled probes by standard procedures (13). DNA size markers were generated by digestion of pBR322 with *Hae*III and labeling of the resulting fragments with [α-^{32}P]dGTP using the Klenow DNA polymerase. RNA size markers were generated by *in vitro* transcription in the presence of [α-^{32}P]CTP of a pre-tRNA^{Gln} from *Synechocystis* with or without the 3′-terminal CCA sequence (7). Primer extensions were performed by standard procedures with total RNA and the oligonucleotides described below labeled with [γ-^{32}P]ATP and polynucleotide kinase.

Primers Used—The following synthetic oligonucleotides were used for PCR or primer extension: 10SAPE, 5′-CTAGGCTGCTATGGCTAC-C-3′; 4.5SPE1, 5′-CTTATTGCTGCTACCTTCCG-3′; 4.5SPE2, 5′-CTA-

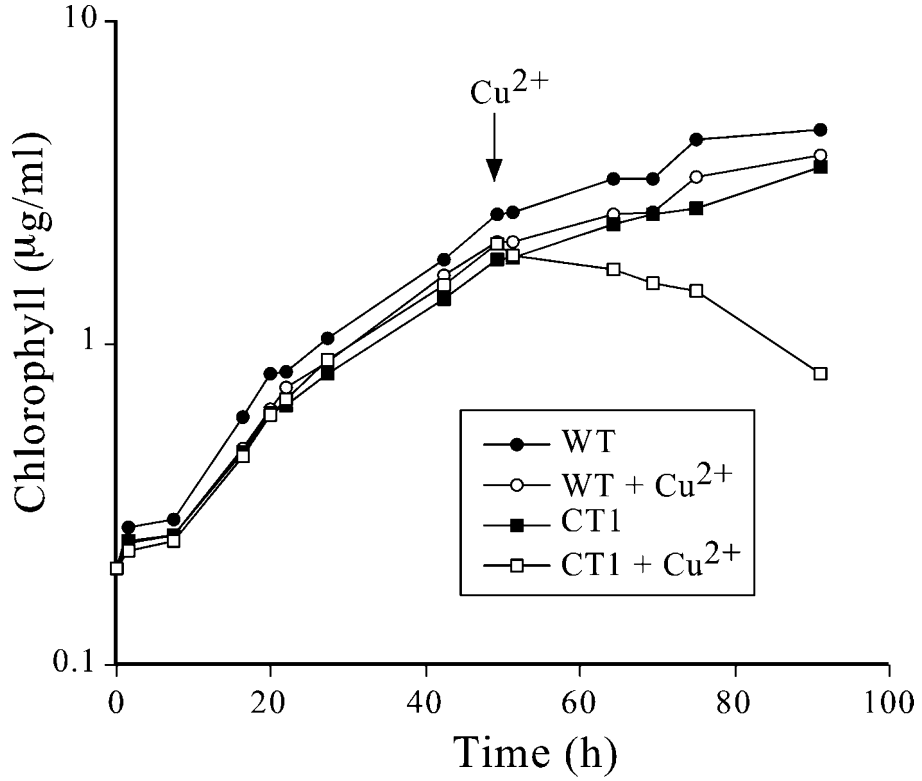

FIG. 3. **Expression of *rnpB* is essential for growth in *Synechocystis*.** Growth curves of the *Synechocystis* wild type and CT1 strains are represented. Duplicate cultures of wild type (*circles*) and CT1 (*squares*) cells were grown on BG11c medium in the absence of copper. At the times indicated, 5 μM $CuSO_4$ was added to one culture of each strain (*open symbols*), while the other culture was maintained in the absence of copper (*black filled symbols*). The initial chlorophyll concentration was 0.2 μg/ml.

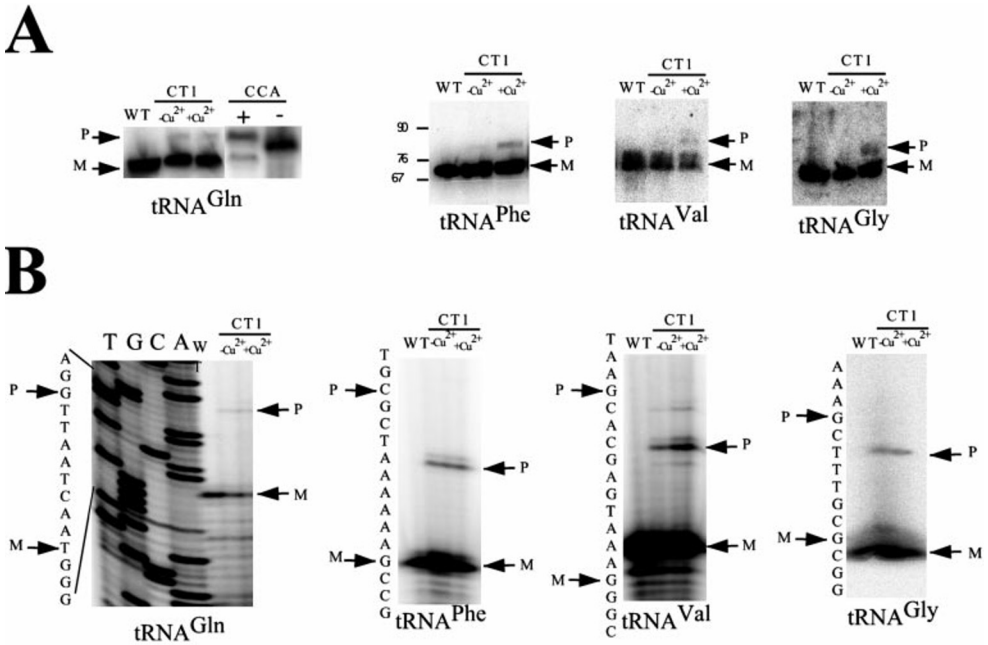

FIG. 4. **Accumulation of 5′-extended precursors of monomeric tRNAs upon RNase P depletion in CT1.** *A*, Northern blots of total RNAs extracted from wild type cells incubated in the presence of Cu^{+2} and from CT1 cells incubated in the absence or in the presence of Cu^{+2}, as indicated. Probes specific for $tRNA^{Gln}$, $tRNA^{Phe}$, $tRNA^{Val}$, and $tRNA^{Gly}$ were used as described under "Experimental Procedures." In the case of $tRNA^{Gln}$, pre-$tRNA^{Gln}$ transcribed *in vitro*, and either containing or lacking the CCA 3′-terminal sequence (7), was used as a marker. The size of pBR322/*Hae*III fragments used as markers for the other tRNAs is indicated. *B*, primer extension of the same RNA samples used for the Northern blots. The oligonucleotides used were GlnR2, trnFr, trnVr, and trnGr for $tRNA^{Gln}$, $tRNA^{Phe}$, $tRNA^{Val}$, and $tRNA^{Gly}$, respectively. The size of the primer-extended products was estimated from a sequencing reaction generated with the same primer run in parallel (shown only for $tRNA^{Gln}$). At least some of the additional bands observed in $tRNA^{Val}$ could be explained by priming of $tRNA^{Ala}$ (see "Experimental Procedures"). *M*, mature RNA; *P*, precursor RNA.

ACCTATCAACACTTTCCGTCG-3′; GlnR2, 5′-CCTAGGAATGGCGGGACC-3′; petf, 5′-TCATAGCAACGCATGCGAGAGTTAGGGAGGG-3′; petJ, 5′-CTAGCTTGGTTGAATAATTTAAACATTAGTTCTC-3′; petr, 5′-TCATACCTCAGGTAACCAAAAAAAGAGAGTTAGTC-3′; PTR3, 5′-GGTATTTTTCTGTGGCACTGTCC-3′; tRYTPE, 5′-GCTGGATTTGAACCAGCGTAGG-3′; trnFf, 5′-CCCATAACCGCTGGC-3′; trnFr, 5′-CGAGGATTTTCAGTCCACTG-3′; trnGf, 5′-CGGATGCATCCCATAGCAGTG-3′; trnGr, 5′-CTAAAGCATGGGAGGCTTTCG-3′; trnVf, 5′-GGCAAAAAGGGAATAGG-3′; trnVr, 5′-CATCCTGCTTGTAAGGCAGGCGC-3′.

Probes Used for Hybridization—The following DNA probes were used in hybridization experiments: rnpB, 1.6-kilobase pair *Hin*dIII fragment from pAV1000 (16); trnQ(UUG), 120-base pair fragment from plasmid pT7Gln (18) digested with *Bam*HI and *Hin*dIII; trnY(GUA)-trnT(GGU), a plasmid containing the gene for the dimeric precursor of $tRNA^{Tyr}$ and

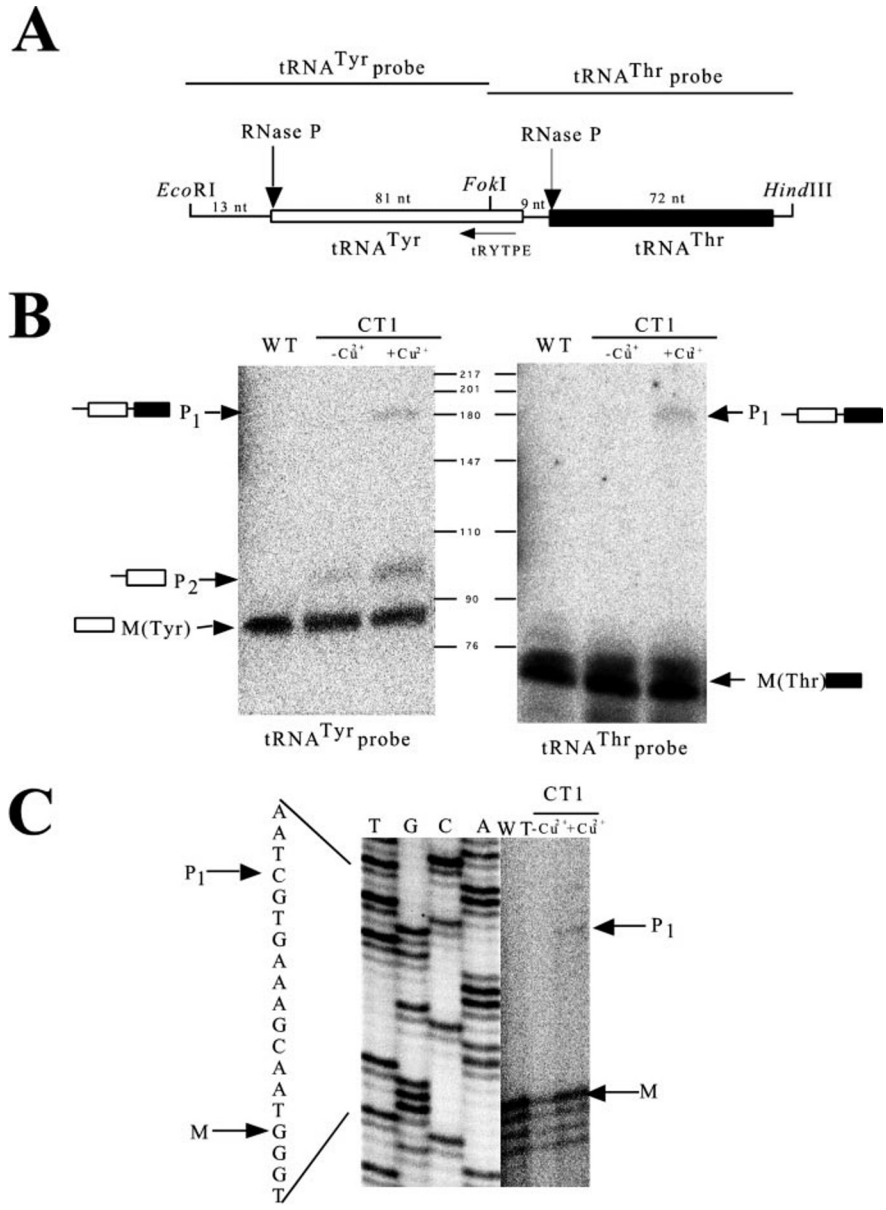

FIG. 5. **Precursors of the dimeric tRNA^{Tyr}-tRNA^{Thr} gene suggest a sequential processing order.** *A*, schematic representation of the dimeric *trnY-trnT* gene and the probes and oligonucleotide used to analyze its transcripts. Lengths in nucleotides (*nt*) are indicated. *B*, Northern blots of total RNAs extracted from wild type cells incubated in the presence of Cu^{2+} and of CT1 cells incubated in the absence or in the presence of Cu^{2+} as indicated. Probes specific for tRNA^{Tyr} and tRNA^{Thr} were used as described under "Experimental Procedures." The size of pBR322/*Hae*III fragments used as markers is indicated. The different precursors detected (P_1 and P_2) and the mature tRNA products (*M*) are represented by the side of the corresponding bands. *C*, primer extension of the same RNA samples used for the Northern blots with oligonucleotide tRYTPE. The sizes of the primer-extended products were estimated from a sequencing reaction generated with the same primer run in parallel. The positions of the 5′ end of the precursor (P_1) and of the mature tRNA^{Tyr} (*M*) are indicated.

tRNA$^{Thr\,2}$ was used to generate a probe specific for the tRNA^{Tyr} by digestion with *Eco*RI and *Fok*I or a probe specific for the tRNA^{Thr} by digestion with *Fok*I and *Hin*dIII (see Fig. 5); trnF(GAA), a PCR fragment was obtained with oligonucleotides trnFf and trnFr. The fragment extends from position −105 to +46 with respect to the first nucleotide of the mature tRNA^{Phe}; trnV(UAC), a PCR fragment was obtained with oligonucleotides trnVf and trnVr. The fragment extends from position −93 to +46 with respect to the first nucleotide of the mature tRNA^{Val}; trnG(CCC), a PCR fragment was obtained with oligonucleotides trnGf and trnGr. The fragment extends from position −175 to +44 with respect to the first nucleotide of the mature tRNA^{Gly}; ffs (4.5 S RNA), a plasmid containing the genomic region around Synechocystis *ffs* gene[2] was used to generate a probe specific for the 4.5 S RNA by digestion with *Dde*I and *Spe*I.

Oligonucleotides and probes were designed based on the published complete sequence of the *Synechocystis* sp. PCC6803 genome (19), available at www.kazusa.or.jp/cyano/cyano.html. Oligonucleotides and probes are specific for their target RNAs, with the exception of oligonucleotide trnVr, which is expected to hybridize significantly with a tRNA^{Ala} and PT3, which has several possible weak homologous regions in the *Synechocystis* genome (see "Results").

[2] M. Roche and A. Vioque, unpublished data.

RESULTS

Construction and Properties of the Synechocystis CT1 Strain—Two sequential steps were followed to create a *Synechocystis* strain with conditional expression of the *rnpB* gene. First, a copy of the *rnpB* gene that was under the control of the copper-regulated *petJ* promoter was placed in the *glnN* locus (Fig. 1*A*). Then, the wild type allele of *rnpB* gene, which is constitutively expressed, was deleted from its regular locus (Fig. 1*B*). The *petJ* gene encodes the cytochrome c_{553} and is tightly repressed by micromolar concentrations of copper (20). Therefore, we expected that the expression of the *rnpB* allele inserted in the *glnN* locus was repressed by copper. The *glnN* locus was selected for the insertion of P_{petJ}-*rnpB* because it is not essential in *Synechocystis* (21). The plasmid pSCT1 was used to insert P_{petJ}-*rnpB* in the *glnN* locus (see "Experimental Procedures"). After transformation with pSCT1, transformants were selected on kanamycin-containing plates and replated several times until segregation was complete, as assessed by Southern blot with a *glnN* probe (data not shown). To inactivate the endogenous *rnpB* gene, one selected pSCT1 transformant was transformed with plasmid pSCT2 (see "Experimental

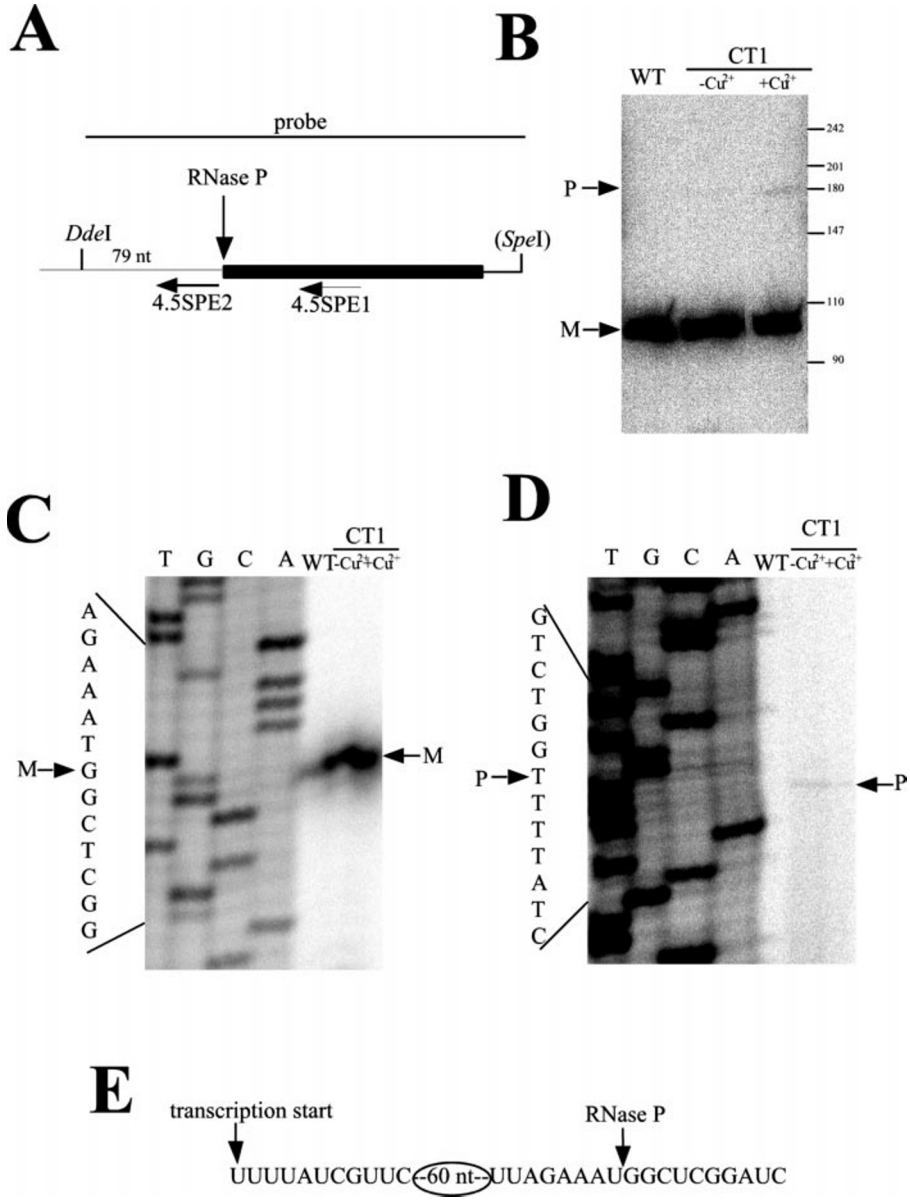

FIG. 6. **RNase P is required for processing of 4.5 S RNA in *Synechocystis*.** *A*, diagram of the *ffs* gene indicating the position of the probe used for Northern hybridization and the oligonucleotides used for primer extension. The *Spe*I site is not genomically encoded; it was originated through the cloning procedure from a plasmid multiple cloning site. *B*, Northern blots of total RNAs extracted from wild type cells incubated in the presence of Cu^{+2} and of CT1 cells incubated in the absence or in the presence of Cu^{+2} as indicated. A probe specific for the 4.5 S RNA was used (see "Experimental Procedures"). Size markers are indicated on the side, as well as the bands corresponding to the precursor and mature RNAs. *C*, primer extension with oligonucleotide 4.5SPE1. *D*, primer extension with oligonucleotide 4.5SPE2 (specific for the pre-4.5 S RNA). The sizes of the primer-extended products were estimated from a sequencing reaction generated with the same primers run in parallel. *E*, sequence of the precursor of 4.5 S RNA around the transcription start point and the RNase P cleavage site. *M*, mature RNA; *P*, precursor RNA.

Procedures"). In this case, transformants were selected in medium without added copper and in the presence of kanamycin and chloramphenicol. Complete segregation of the double mutant (named CT1) was obtained after several rounds of replating. Southern blot of the CT1 strain with an *rnpB* probe revealed no detectable hybridization band corresponding to the wild type *rnpB* gene. Only the bands corresponding in size to the chloramphenicol-replaced *rnpB* gene and the P_{petJ}-*rnpB* gene at the *glnN* locus were detected (Fig. 1*C*).

The expression of *rnpB* was analyzed in the wild type and in the CT1 strains by primer extension (Fig. 2). The transcript synthesized from the *petJ* promoter has 39 additional nucleotides at the 5′ end. This is the result expected if the P_{petJ}-*rnpB* gene fusion uses the same transcription start point as the *petJ* gene (determined by primer extension with a *petJ*-specific oligonucleotide; data not shown). Transcription from the *rnpB* locus is not observed in CT1. Fig. 2 shows that transcription of *rnpB* from the P_{petJ} promoter is fully repressed by copper in CT1. However, an additional band of unknown origin is observed in the CT1 strain. We attribute this band to nonspecific priming during the primer extension procedure. When the amount of RNase P RNA is reduced, competition by a nonspecific priming site would be facilitated. This could explain why the band is more abundant in the copper-treated CT1 cells, where no RNase P RNA-specific signal is detected.

There is no indication that there is processing at the 3′ end of the normal *rnpB* transcript, and transcription seems to terminate at the mature 3′ end (16). We have not characterized in detail the 3′ end of the P_{petJ}-*rnpB* transcript, but Northern blot (not shown) indicates that the P_{petJ}-*rnpB* transcript is only slightly longer than the normal *rnpB* transcript. The difference in size is fully explained by the 39 extra nucleotides at the 5′ end, suggesting that the P_{petJ}-*rnpB* transcript has the same 3′ end as the normal *rnpB* transcript.

We anticipated that the RNase P RNA carrying a short segment of additional nucleotides would be functional, because *Synechocystis* RNase P RNAs with longer extensions at the 5′ or 3′ ends had been shown to be functional *in vitro* (16) and because RNase P activity can be detected even when the RNase P RNA is part of a much larger RNA generated by rolling circle transcription (22). The fact that the *rnpB* gene could be fully segregated in the CT1 strain indicates that the RNA transcribed from the *petJ-rnpB* site, with the 39 additional nucleotides at the 5′ end, is functional *in vivo*. The steady-state levels of RNase P protein present in the wild type and CT1 strains in the presence or absence of copper did not change

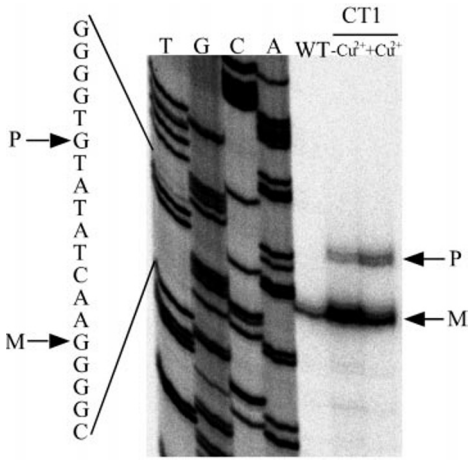

FIG. 7. **RNase P is required for processing of tmRNA in *Synechocystis*.** Primer extension of total *Synechocystis* RNA with primer 10SaPE specific for the tmRNA is shown. The sizes of the primer-extended products were estimated from a sequencing reaction generated with the same primer run in parallel. *M*, mature RNA; *P*, precursor RNA.

significantly, as determined by Western blot (not shown) with antibodies generated against the RNase P protein of *Synechocystis* (18).

Fig. 2 shows that the transcription level of the RNase P RNA is lower in CT1 grown in the absence of copper than in the wild type strain. However, it is enough to support normal growth of the cells, because in the absence of copper, strain CT1 grows at a rate similar to wild type (Fig. 3). Therefore, we conclude that under fully derepressed conditions of the *petJ* promoter (absence of copper) the steady-state levels of RNase P in CT1 cells are not limiting growth, even though the amount of RNase P RNA is lower than in wild type. The addition of 5 μM copper has no effect on wild type growth, whereas it inhibits the growth of CT1. The addition of copper results in repression of the *petJ* promoter and reduction of the amount of RNase P RNA and growth inhibition. We conclude that the RNase P RNA is required for *Synechocystis* survival.

5′-Extended Pre-tRNAs Accumulate in CT1—Transcripts from four different monomeric tRNA genes, *trnQ(UUG)*, *trnF-(GAA)*, *trnV(UAC)*, and *trnG(CCC)*, coding, respectively, for $tRNA^{Gln}$, $tRNA^{Phe}$, $tRNA^{Val}$, and $tRNA^{Gly}$ species were analyzed by Northern blot and primer extension. The Northern blot experiments (Fig. 4*A*) showed a single band with a size corresponding to the mature tRNA in the wild type strain. In the CT1 strain, an additional band of larger size was observed upon depletion of RNase P. In principle, this larger band could correspond to precursor RNAs not fully processed at the 5′ or 3′ ends. Primer extension experiments with oligonucleotides specific for each tRNA indicate the presence of 5′-extended tRNAs (Fig. 4*B*). The larger total size of the precursors detected by Northern blot were fully explained by the extension at the 5′ end, indicating that those precursors were already processed at their 3′ end. In the case of $tRNA^{Gln}$ we were able to determine that the precursor detected has a size that corresponds exactly to an *in vitro* transcribed precursor with a processed 3′ end and containing the 3′-terminal CCA sequence. Therefore, it is probable that the precursors detected are already processed at the 3′ end, have the CCA added, and the increased size corresponds to the absence of 5′ processing by RNase P, as expected if expression of *rnpB* is limited. Alternatively, the final trimming of the three nucleotides that occupy the position of CCA might require previous processing by RNase P.

Some precursor RNAs are observed in the CT1 strain even in the absence of copper. This can be explained by the weaker expression of the *petJ* promoter, even under full derepression compared with the native *rnpB* promoter. Therefore, RNase P activity could be limiting for the processing of a subset of its substrates in strain CT1, even in the absence of copper from the medium, although this has no significant effect on growth rate.

Sequential RNase P Processing of the Dimeric Pre-$tRNA^{Tyr}$-$tRNA^{Thr}$ Transcript—In *Synechocystis*, there are 42 tRNA genes all present as single copies, with the exception of the *trnI(GAU)*, which is present in two copies in the duplicated rRNA gene clusters. The genes are scattered all over the genome as single units except for *trnY(GUA)* and *trnT(GGU)*, which are separated by only 9 base pairs (23). Maturation of this dimeric precursor would require cleavage by RNase P at two positions, corresponding to the mature 5′ ends of $tRNA^{Tyr}$ and $tRNA^{Thr}$, respectively (Fig. 5*A*). When RNAs were analyzed by Northern blot with a $tRNA^{Tyr}$-specific probe (Fig. 5*B*), two bands of larger size than mature $tRNA^{Tyr}$ were detected. One band, of about 180 nucleotides, corresponds to the dimeric precursor already processed at the 3′ end of $tRNA^{Thr}$ but with no processing by RNase P. The other band corresponds to a precursor RNA already processed at the RNase P site of $tRNA^{Thr}$. However, with a $tRNA^{Thr}$-pecific probe, only the 180-p band was detected. No band corresponding to RNase P processing at only the $tRNA^{Tyr}$ site was observed, indicating that processing at the downstream $tRNA^{Thr}$ site must precede RNase P cleavage at the $tRNA^{Tyr}$ site.

5′-Extended Precursors for 4.5 S RNA and tmRNA Accumulate in CT1—In *E. coli*, RNase P is involved in the 5′ processing of some stable RNAs such as 4.5 S RNA and tmRNA (8, 9). To find out if *Synechocystis* RNase P is also involved in the processing of these RNAs, we determined whether precursors for those RNAs were accumulated upon addition of copper to cultures of CT1 cells. In the case of 4.5 S RNA, a band of about 180 nucleotides was detected in CT1 by Northern hybridization (Fig. 6*B*). This band may correspond to a 5′-extended RNA that could be detected by primer extension with a primer specific for the leader sequence (Fig. 6*D*). The precursor has a leader sequence of 79 nucleotides (Fig. 6*E*). This precursor seems to be very unstable, because it could only be detected with an oligonucleotide specific for the leader, and the signal was very weak (Fig. 6, *C* and *D*). In contrast, a strong signal for a precursor for tmRNA was detected by primer extension in CT1 (Fig. 7). In this case, the leader sequence is 11 nucleotides long (Fig. 7). These results indicate that 4.5 S RNA and tmRNA are synthesized as precursors that are processed by RNase P in *Synechocystis*.

Promoters of tRNA Genes—The mapping that we have performed of the 5′ end of pre-tRNAs provides the transcription start point for the corresponding tRNA genes. We have compared the putative promoter sequences of those genes for which we have mapped the 5′ end of the precursors (Fig. 8). −10 and −35 consensus sequences are detected at the appropriate distance from the first nucleotide of the precursor, although in the case of *trnV*, *trnG*, and 4.5 S RNA, the −35 sequence has a poor agreement with the consensus. In the 4.5 S RNA gene there is a heptanucleotide direct repeat with a 5-base pair spacer just 4 base pairs upstream of the proposed −10 sequence.

DISCUSSION

The high efficiency of RNA processing events in bacteria makes it very difficult to detect precursors or intermediates in the maturation process in conditions of steady state. The same is true for pre-tRNA. Pre-tRNAs were first detected in phage-infected cells (24) after quick phenol RNA extraction. In addition, conditional mutants in tRNA maturation have been useful in the detection of the corresponding precursors (25). A strain with conditional expression of the *rnpB* gene was developed in

FIG. 8. **Comparison of the promoter region of the genes studied.** The 5′ sequences of *trnQ*, *trnF*, *trnV*, *trnG*, *trnY-trnT*, *ssrA*, and *ffs* are shown and aligned at the 5′ end of the precursor RNA (*bold uppercase*). The sequences end at the first nucleotide of the mature RNA (*italics*). In the case of 4.5 S RNA, the mature RNA starts 79 nucleotides downstream of the 5′ end of the precursor RNA. Possible −10 and −35 hexanucleotides are *underlined*. A direct repeat of the sequence GAAATGC is shown in *bold* in the 4.5 S RNA gene.

E. coli (26), although it was not used for the study of the maturation pathway *in vivo*. In this report, we have used a *Synechocystis* strain that expresses conditionally the *rnpB* gene to identify precursors in the maturation process of transfer and other stable RNAs. We have followed an original approach to isolate a mutant of *Synechocystis* 6803 that expresses conditionally the *rnpB* gene from the *petJ* gene promoter, depending on the concentration of copper in the medium. This approach, using plasmid pSCT1 as a platform, can be used to obtain conditional mutants affected in other essential genes. A recent report (27) describes the conditional expression of a regulatory gene from the cyanobacterium *Anabaena* under the control of another copper-regulated promoter, the *petE* gene promoter. The regulation of *petE* is complementary to *petJ*, because it is expressed only in the presence of copper. Therefore, conditional expression form the *petE* or *petJ* promoters represents complementary systems that can be used in diverse cyanobacteria.

The analysis of the conditional *rnpB* mutant has allowed us to identify and characterize 5′-extended pre-tRNAs. In five arbitrarily chosen tRNAs, we could detect 5′-extended precursors, as well as in other stable RNAs that are expected to be processed by RNase P, such as tmRNA and 4.5 S RNA.

The 5′ ends determined for the precursor RNAs most probably correspond to the transcription initiation site. There are two arguments in favor of this idea. First, only RNase P has been implicated in 5′ maturation of pre-tRNAs that are not part of complex transcripts. Second, a sequence similar to the consensus −10 box is present at the appropriate distance of the precursor 5′ end; in most cases a canonical −35 box is also present (Fig. 8).

The length of the leader sequences detected are rather short, around 10 nucleotides, with the exception of 4.5 S RNA, that has a leader sequence of 79 nucleotides. This is in agreement with the known fact that bacterial RNase P is highly tolerant with respect to size and sequence of the 5′ leader.

The 5′-extended pre-tRNA detected in strain CT1 seems to be already processed at the 3′ end, because the size of the precursors determined by Northern blot correspond to that of the mature tRNA plus the 5′ leader sequence. These results indicate that there is a quick and efficient 3′ end processing, which is independent of RNase P activity. Do the 3′ processed precursors contain the 3′-CCA sequence? We have previously described (7) that cyanobacterial RNase P has a preference *in vitro* for pre-tRNAs lacking the 3′-CCA sequence (in *Synechocystis*, the 3′-CCA sequence is not encoded, but is added post-transcriptionally by nucleotidyltransferase). This prompted us to suggest that RNase P processing could precede addition of CCA in cyanobacteria (7). In the case of $tRNA^{Gln}$, we could compare the size of the *in vivo* accumulated precursor with the size of *in vitro* transcribed pre-$tRNA^{Gln}$ containing or lacking the CCA sequence (Fig. 4*A*). Clearly, the *in vivo* precursor comigrates with the CCA containing marker RNA and not with the CCA-lacking RNA. This result suggests that CCA addition by tRNA nucleotidyltransferase can occur *in vivo* before RNase P processing. However, the dominant order of events cannot be deduced from these results. RNase P processing might precede CCA addition under normal conditions in the wild type, despite the fact that in CT1, upon limitation of RNase P, the accumulated 5′ precursors are substrates of the nucleotidyltransferase. Alternatively, RNase P and nucleotidyltransferase might act on the pre-tRNA independently, giving a random processing pathway. A strain with conditional expression of nucleotidyltransferase may solve these questions.

In the absence of copper, some accumulation of precursors is detected, indicating that the lower constitutive level of RNase P RNA in CT1, compared with wild type, is already limiting processing. The fact that the growth rate of CT1 in the absence of copper is similar to wild type indicates that accumulation of precursors is not a secondary effect of growth arrest, but a specific consequence of RNase P depletion.

In the case of the only dimeric pre-tRNA in *Synechocystis*, our results indicate an obligate pathway of RNase P processing. The downstream site is used first. Processing at the upstream site cannot happen until the downstream tRNA has been released, because precursors processed only at the upstream site are not detected. A similar *in vivo* processing order was deduced for a dimeric precursor of a T4 $tRNA^{Pro}$-$tRNA^{Ser}$ in phage-infected *E. coli* cells (28). However, in this case the 3′ monomer has encoded the CCA sequence.

Even after 48 h in the presence of copper, most of the RNAs are in the mature form, and only a small proportion of RNase P precursor is detected. This could be due to a longer half-life of mature tRNAs compared with their precursors. But it is interesting that growth arrest is immediate upon addition of copper even though the amount of mature tRNA in CT1 cells is similar in cultures with or without added copper. This might indicate that there is an unknown RNase P-dependent substrate whose abundance is critical for cell growth that has not been revealed in our study or that the accumulated precursors poison the cell despite the presence of a sufficient amount of properly processed RNAs.

It is possible that there are other natural substrates for bacterial RNase P yet to be identified. Those hypothetical substrates might be difficult to identify if they are in low abundance, are present only under specific metabolic conditions, or are cryptic and only accessible to RNase P after some previous modification of the RNA. The construction of a strain in which expression of RNase P can be shut down under controlled experimental conditions could help identify these substrates.

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