Genes Encoding the Group I Intron-containing tRNA^Leu^ and Subunit L of NADH Dehydrogenase from the Cyanobacterium *Synechococcus* PCC 6301

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Abstract

A part of the tRNA^Leu^ (UAA) gene containing a 240-nucleotide group I intron was amplified by PCR from cyanobacterium *Synechococcus* PCC 6301 genomic DNA. The pre-tRNA synthesized from the cloned PCR product was efficiently self-spliced in vitro under physiological conditions. The gene encoding the tRNA^Leu^ (UAA), *trnL-UAA*, was isolated from a *Synechococcus* PCC 6301 genomic library and the nucleotide sequence of a 2,167-bp portion was determined. The *trnL-UAA* consists of a 34-bp 5' exon, a 240-bp group I intron and a 50-bp 3' exon. In addition, three open reading frames (ORF1, ORF2 and ORF3) were found in the 5' and 3' flanking regions of *trnL-UAA*. The predicted protein sequence of ORF3, which is located 74-bp upstream from *trnL-UAA* on the opposite strand, shows 66.2% amino acid identity to that of the *Synechocystis* PCC 6803 gene encoding subunit L of NADH dehydrogenase (*ndhL*).

Key words: *Synechococcus* PCC 6301; group I intron; tRNA^Leu^; self-splicing; *ndhL*

1. Introduction

Cyanobacteria are autotrophic bacteria capable of oxygenic plant-type photosynthesis and are thought to be the progenitor of chloroplasts. tRNA^Leu^ (UAA) genes containing a group I intron exist in chloroplasts, cyanelle and cyanobacteria and their intron sequences are highly conserved. So far, group I intron-containing tRNA^Leu^ genes have been found in five cyanobacterial species, including *Anacystis nidulans* R2 (*Synechococcus* PCC 7942). The tRNA^Leu^ intron of the multicellular cyanobacterium *Anabaena azollae* was shown to self-splice during in vitro transcription. Recently, Anug et al. have demonstrated that *Anabaena* PCC 7120 pre-tRNA^Leu^ self-splices in vitro. However, it is not known whether the *Synechococcus* tRNA^Leu^ intron splices in vitro and its entire gene structure remains to be analyzed.

Here, we present evidence for in vitro self-splicing of the group I intron of pre-tRNA^Leu^ from *Synechococcus* PCC 6301, a strain closely related to *Synechococcus* PCC 7942. In addition, the gene encoding tRNA^Leu^ (UAA) was isolated and characterized.

2. Materials and Methods

2.1. Isolation of *Synechococcus* genomic DNA

Genomic DNA from the unicellular cyanobacterium *Synechococcus* PCC 6301 (formerly *Anacystis nidulans* 6301) was prepared as described.

2.2. Amplification of the *Synechococcus* PCC 6301 group I intron

PCR amplification was carried out using the following primers: L5 (5'-GGAAGCTTGGTGTGGCGGAATGGTAGAC) which corresponds to positions 4-23 of *A. azollae* tRNA^Leu^, and L3 (5'-GGAAGCTTGGAATGGTGACCGCTTGAAATG) which corresponds to positions 4-23 of *A. azollae* tRNA^Leu^ and L3 (5'-GGAAGCTTGGAATGGTGACCGCTTGAAATG) which corresponds to positions 4-23 of *A. azollae* tRNA^Leu^.

The amplified DNA fragment was digested with *HindIII* sites are underlined. PCR was performed for 30 cycles according to the manufacturer's instructions (Perkin-Elmer Cetus, USA); each cycle consisted of treatments at 94°C for 1 min, 55°C for 1.5 min and 72°C for 1.5 min (for 7 min in the last cycle). The amplified DNA fragment was digested with *HindIII*, purified in a 1.5% agarose gel and ligated with *HindIII*-digested pBluescript SK(+) ver. II. The orientation of insert DNAs of the resultant plasmids, pTL3 and pTL7, are opposite. The DNA sequence was verified by double-strand sequencing by the dideoxy chain termination method (Sequenase, US Biochemical).
Figure 1. Self-splicing of pre-tRNA\textsubscript{Leu} (UAA). (A) Products of \textit{in vitro} transcription from linearized pTL3 and pTL7 DNAs. Labels indicate positions expected for the runoff transcript (Pre), linear intron (LI) and ligated exons (LE). Numbers in parenthesis are in nucleotides (nts). (B) Self-splicing products from purified \textsuperscript{32}P-labeled pre-tRNA. Pre-tRNA of pTL7 was incubated in the presence of 0.2 mM GTP, 10 mM MgCl\textsubscript{2} and 50 mM NH\textsubscript{4}Cl at 25°C (lane 1), 37°C (lane 2) or 45°C (lane 3). The pre-tRNA was incubated in the above solution except for the removal of GTP (lane 4), NH\textsubscript{4}Cl (lane 5) or MgCl\textsubscript{2} (lane 6). Lane 7 shows the result of incubation of pre-RNA in water. Size markers are \textit{Hind}III digests (nts 162-770) of \phi X174 RF-DNA.

2.3. \textit{Self-splicing of group I intron}

pTL3 and pTL7 were linearized with \textit{XhoI} and \textit{XbaI}, respectively. Transcription was carried out at 37°C for 1 h in 40 mM Tris-HCl, pH 8.0, 5 mM MgCl\textsubscript{2}, 5 mM DTT, 2 mM spermidine, 0.5 units of Inhibit-ACE, 0.5 mM each of ATP, GTP, CTP and 0.1 mM UTP, 0.148 MBq [\textsuperscript{32}P]UTP (sp. act. 30 TBq/mmol), 1 \mu g linearized plasmid and 50 units of T3 RNA polymerase (for pTL3) or T7 RNA polymerase (for pTL7). Transcription of pTL3 produced a pre-tRNA of 416 nts, consisting of a 117-nt 5' exon (37-nt insert and 80-nt vector sequences), a 240-nt intron and a 59-nt 3' exon (40-nt insert and 19-nt vector sequences). A transcript of pTL7 is 401-nt, consisting of an 84-nt 5' exon (37-nt insert and 47-nt vector sequences), a 240-nt intron and a 77-nt 3' exon (40-nt insert and 37-nt vector sequences). Synthesized pre-tRNAs were purified by electrophoresis on a 5% polyacrylamide/7 M urea gel and then dissolved in water. Gel-purified pre-tRNA (5,000 cpm) was incubated for 60 min in a 20-\mu l mixture containing 0.2 mM GTP, 10 mM MgCl\textsubscript{2} and 50 mM NH\textsubscript{4}Cl at various temperatures. Then the reaction was terminated by adding an equal volume of stop solution (100 mM EDTA, 1 \mu g \textit{E. coli} tRNA and 4 M NH\textsubscript{4}Cl) and 100 \mu l cold ethanol. Spliced products were separated on a 5% polyacrylamide/7 M urea gel and detected by autoradiography.

2.4. \textit{Screening of a gene encoding tRNA\textsubscript{Leu}}

A \textit{Synechococcus} PCC 6301 genomic DNA library constructed in \textit{AEMBL4}\textsuperscript{a} was screened by plaque hybridization with the insert DNA of pTL7. Hybridization, washing and detection of positive plaques were performed according to the procedure of ECL random prime labelling and detection systems (Amersham). The DNA of the positive phage was prepared and subjected to Southern blot analysis for subcloning as described.\textsuperscript{10,11} A 2.1-kb
The nucleotide sequence of 2,167 bp region in A19. The 5' and 3' exons of trnL-UAA are boxed. Dashed box indicates an intron. P, Q, R, and S are the conserved sequences of group I introns. 12 ORF1, ORF2 and ORF3 are shown from the putative translation initiation codons (underlined) to stop codons (dotted underlined). These ORFs are encoded on the strand opposite trnL-UAA.

The deduced amino acids of ORF3 are italicized. The sequence has been deposited in DDBJ, GSDB, EMBL and NCBI nucleotide sequence databases (accession no. D42186).

EcoRI fragment hybridized to the probe was subcloned into pBluescript II SK(+) plasmid (Stratagene) and both strands were sequenced by a DNA sequencer (Perkin-Elmer, ABI 373A).

3. Results and Discussion

3.1. Self-splicing of the Synechococcus PCC 6301 group I intron

The 317-bp DNA was amplified using primers specific for the tRNALeu (UAA) and subcloned into pBluescript SK(+) to create pTL3 and pTL7. The insert DNAs of pTL3 and pTL7 consisted of a 37-bp 5' exon, a 240-bp group I intron and a 40-bp 3' exon. The nucleotide sequence of the 240-bp intron is identical to that of Ana- cystis nidulans R25. When the linearized pTL3 or pTL7 DNAs were transcribed, three discrete RNA fragments were produced (Fig. 1A). Based on their sizes, the longest ones are pre-tRNAs, middle-sized bands correspond to a 240-nt linear intron (LI) and the faster-migrating products to ligated exons (LE). Similar self-splicing during in vitro transcription was observed in A. azollae pre-tRNALeu.5

To confirm whether these RNA species are produced by self-splicing, we carried out further analysis using the gel-purified pre-tRNA from pTL7. RNAs of the same size were detected under in vitro self-splicing conditions (Fig. 1B). GTP and MgCl₂ were required for self-splicing (Fig. 1B, lanes 4 and 6). Surprisingly, the Synechococcus PCC 6301 group I intron self-spliced at physiological temperature of 25°C (Fig. 1B, lane 1) and in the absence of salts.
Figure 3. Secondary structure of the group I intron of pre-tRNA^{Leu} (UAA). Exon and intron sequences are in lower and in upper cases, respectively. Underlined exon sequences indicate the regions of base-pairing in the tRNA^{Leu} anticodon stem. The 5' and 3' splice sites are indicated by arrows. The conserved sequences, P to S, are indicated by bold letters. Paired regions P1-P9 are indicated.

ORF3 protein

MTVTLLAALYLAALAGAYLLVVPAALAYLYLQLQKWYASSWERAFAFWYLFFFPGLLPLLAPLNFRPRSRQIPA 74

MEDLLGSLSLSETGLLAIYLGSLAYLVFPALLYWYGLQKWYVASSVERLVMYFLFGLPPGLVLSPVLNLRRPR-RQ-AA 80

ndhL protein

Figure 4. Alignment of amino acid sequences deduced from ORF3 and ndhL of Synechocystis PCC 6803.15,16 Asterisks indicate identical residues and dots indicate homologous amino acid replacement. Dashes denote gaps.
(Fig. 1B, lane 5). These results suggest that this group I intron can efficiently splice under normal cell growth conditions at 30°C or less.

Since the PCR products contain only part of the tRNA<sup>Leu</sup> sequence, the entire tRNA coding sequence may not be required for self-splicing. It appears likely, however, that its anticodon stem-loop structure is needed for efficient self-splicing. The efficiency of self-splicing of the pTL3 and pTL7 transcripts differ significantly (Fig. 1A), suggesting that exon sequence and its length affect the rate of splicing of the pre-tRNA in vitro. It is interesting to note that splicing of Anabaena PCC 7120 pre-tRNA<sup>Leu</sup> has been suggested to require the collaboration of tRNA and group I intron structures.

3.2. The tRNA<sup>Leu</sup> (UAA) gene

To isolate and characterize the gene encoding the tRNA<sup>Leu</sup> (UAA), we screened a Synechococcus PCC 6301 genomic DNA library with the insert DNA of pTL7. A λ19 clone containing a 13-kb genomic DNA fragment was isolated and the EcoRI fragment hybridized to the probe was sequenced (Fig. 2). It contains the tRNA<sup>Leu</sup> (UAA) gene, trnL-UAA, and three open reading frames, ORF1, ORF2 and ORF3. The intron is 240-bp and is identical to that of the PCR product, and its secondary structure is shown in Fig. 3. The 5'-tRNA<sup>Leu</sup> exon is 34 bp and the 3' exon is 50 bp. The predicted tRNA<sup>Leu</sup> (UAA) sequence shows 87.1%, 84.7% and 81.2% homology with those of Azolla,<sup>5</sup> cyanelle<sup>1</sup> and tobacco chloroplast<sup>13</sup>. The Synechococcus trnL-UAA gene does not encode a 3'-CCA stem, similar to the Synechococcus PCC 6301 tRNA<sup>Leu</sup>, tRNA<sup>Ala</sup>, and tRNA<sup>Val</sup> genes.<sup>11,12</sup> This is the first report on the group I intron-containing tRNA<sup>Leu</sup> gene from the unicellular cyanobacterium Synechococcus sp. The trnL-UAA gene is present as a single-copy gene and is mapped between trnA and trnB on the Synechococcus PCC 6301 chromosome (unpublished data).

3.3. The gene encoding subunit L of NADH dehydrogenase

The deduced amino acid sequences of ORF1 (longer than 128 codons) and ORF2 (138 codons) do not correspond to any known polypeptides deposited in protein sequence databases (Swiss Prot Rel. 29 and NBRF-PDB Rel. 40). ORF3 is located 74 bp upstream from trnL-UAA on the opposite strand. A homology search revealed that the predicted amino acid sequence of ORF3 (74 codons) shows 66.2% amino acid identity and 83.7% amino acid similarity to that of the unicellular cyanobacterium Synechocystis PCC 6803 gene for subunit L of NADH dehydrogenase, ndhL. (15; formerly ictA, ref. 16), therefore we identified ORF3 as ndhL. (Fig. 4). No trnL gene is located within 420 bp upstream from ndhL in Synechocystis PCC 6803.<sup>15,16</sup> Putative "-10" and "-35" sequences are located in its upstream region. Putative promoter sequences of trnL and ndhL are overlapped on opposite strands (Fig. 2). If true, this is the first example of a divergent promoter in cyanobacterial genes. This suggests the existence of a unique promoter motif for trnL.

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References


13. Yamada, K., Shinozaki, K., and Sugiura, M. 1986, DNA sequences of tobacco chloroplast genes for tRNA^{Ser} (GCA), tRNA^{Thr} (UGU), tRNA^{Leu} (UAA), tRNA^{Phe} (GAA); the tRNA^{Leu} gene contains a 503 bp intron, *Plant Mol. Biol.*, 6, 193–199.

