Expression of the Synechocystis sp. Strain PCC 6803 tRNA\textsubscript{Glu} Gene Provides tRNA for Protein and Chlorophyll Biosynthesis

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In the cyanobacterium Synechocystis sp. strain PCC 6803 (Synechocystis 6803) \&-aminolevulinic acid (ALA), the sole precursor for the synthesis of the porphyrin rings of heme and chlorophyll, is formed from glutamate activated by acylation to tRNA\textsubscript{Glu} (G. P. O'Neill, D. M. Peterson, A. Schön, M. W. Chen, and D. Söll, J. Bacteriol. 170:3810–3816, 1988; S. Rieble and S. I. Beale, J. Biol. Chem. 263:8864–8871, 1988). We report here that Synechocystis 6803 possesses a single tRNA\textsubscript{Glu} gene which was transcribed as monomeric precursor tRNA and matured into the two tRNA\textsubscript{Glu} species. They differed in the extent of modification of the first anticodon base, 5-methylaminomethyl-2-thiouridine (O'Neill et al., 1988). The two tRNA species had equivalent capacities to stimulate the tRNA-dependent formation of ALA in Synechocystis 6803 and to provide glutamate for protein biosynthesis in an Escherichia coli-derived translation system. These results are in support of a dual role of tRNA\textsubscript{Glu}. The levels of tRNA\textsubscript{Glu} were examined by Northern (RNA) blot analysis of cellular RNA and by aminoacylation assays in cultures of Synechocystis 6803 in which the amount of chlorophyll synthesized was modulated over a 10-fold range by various illumination regimes or by the addition of inhibitors of chlorophyll and ALA biosynthesis. In these cultures, the level of tRNA\textsubscript{Glu} was always a constant fraction of the total tRNA population, suggesting that tRNA\textsubscript{Glu} and chlorophyll levels are regulated independently. In addition, the tRNA\textsubscript{Glu} was always fully aminoacylated in vivo.

An abundant supply of aminoacyl-tRNA is vital to ensure optimal protein biosynthesis in response to a cell’s changing needs. A long-standing question has been how the level of aminoacyl-tRNA is regulated. This is especially important in cells which have specialized to make preferentially a particular protein or in cells responding to changes in metabolic or environmental conditions. In such cases the steady-state concentration of specific tRNA species and aminoacyl-tRNA synthetases can be "functionally adapted" to match a new cellular requirement (22, 23). The best-described example of specialized cells is the Bombyx mori posterior silk gland, which makes fibroin, a protein of very unusual amino acid composition (24). In the silk gland, the tRNA pool is specifically enriched with tRNA species cognate for the amino acids (e.g., Ala) found in fibroin (26). It is now known that the higher level of tRNA\textsubscript{Ala} is due to tissue-specific expression of a special tRNA\textsubscript{Ala} gene (68). A second situation in which alterations of tRNA concentrations and aminoacyl-tRNA synthetases are made to match cellular requirements is during certain metabolic conditions such as amino acid limitation (14, 42, 61). No mechanism has been elucidated for this case.

We were intrigued by the possibility that functional adaptation of a specific tRNA species and its cognate aminoacyl-tRNA synthetase may also occur during chlorophyll synthesis for the tRNA-dependent formation of \&-aminolevulinic acid (ALA), the precursor for the porphyrin rings of chlorophyll and heme. As shown in Fig. 1, there are two well-characterized pathways for the formation of ALA (for reviews, see references 6 and 33). In the Shemin pathway, succinyl-coenzyme A and glycine are condensed to ALA by the enzyme ALA synthase. This route of ALA synthesis is present in mammals, yeasts, and certain procaryotes, including Rhodopseudomonas and Rhizobium species (12). A second route of ALA synthesis, the C5 pathway, involves the enzymatic transformation of glutamate into ALA by a multistep, tRNA-dependent mechanism (33). The C5 pathway, originally detected in the chloroplasts of higher plant (5, 33) and algal (31) species, appears to be present in a wide variety of eubacterial species (3), including Escherichia coli (2, 39, 47), Bacillus subtilis (47), and archaebacteria (21).

ALA formation in Synechocystis sp. strain PCC 6803 (Synechocystis 6803) is remarkably similar to that found in plant and algal chloroplasts (33, 48, 51). In the first step of the pathway (Fig. 1), glutamate is esterified to RNA\textsubscript{Glu} by glutamyl-tRNA synthetase (GluRS). Glutamate is then reduced by Glu-tRNA reductase to yield glutamate-1-semialdehyde. ALA is formed in a final transamination step, catalyzed by glutamate-1-semialdehyde aminotransferase.

We had shown earlier that Synechocystis 6803 has two tRNA\textsubscript{Glu} species which can be aminoacylated and which support RNA-dependent ALA formation in vitro (48). The two tRNA\textsubscript{Glu} species have identical primary sequences but differ in the extent of modification of the first anticodon base, 5-methylaminomethyl-2-thiouridine (48). The involvement of the Synechocystis tRNA\textsubscript{Glu} species in a non-protein-biosynthetic process raised a number of intriguing questions. Are the intracellular tRNA\textsubscript{Glu} levels altered under different conditions of photosynthetic growth when demand for chlorophyll and ALA increases or decreases? Are there multiple tRNA\textsubscript{Glu} genes providing tRNA\textsubscript{Glu} either for protein biosynthesis or for ALA biosynthesis? Do such genes possess features different from other tRNA genes? Can the same tRNA\textsubscript{Glu} species function in both ALA formation and protein biosynthesis? In this report we attempt to answer these questions by examining the structure and expression of the Synechocystis 6803 tRNA\textsubscript{Glu} gene and the use of its tRNA product.

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MATERIALS AND METHODS

Materials. E. coli tRNA\textsubscript{Glu} (38) was originally obtained from G. D. Novelli of the Oak Ridge National Laboratory. Uniformly labeled L-\textsuperscript{14}C-amino acids were obtained commercially. Their specific activities (in millicuries for milliliter) are: alanine, 10; arginine, 10; glycine, 98.8; glutamate, 293; isoleucine, 10; leucine, 10; phenylalanine, 485; proline, 10; and serine, 10. The plasmid pTAC101-DHFR\textsubscript{wt}, which contains the \textit{E. coli} dihydrofolate reductase (DHFR) gene on a 1,057-bp \textit{HindIII} fragment, was obtained from J. Normanly (44). Gabaculin (3-amino-2,3-dihydrobenzoic hemihydrate), ALA, hemin, levulinic acid, and DE32 and DE52 anion-exchange resins were obtained commercially.

Strains, media, and growth conditions. \textit{E. coli} JP1449 (obtained from J. Lapointe) is a thermosensitive strain containing a thermolabile GluRS (37, 53). \textit{E. coli} XL-1 was from Stratagene, La Jolla, Calif. (11). \textit{Synechocystis} 6803 (obtained from A. Y. Cheung) was grown photoautotrophically or photoheterotrophically (0.2\% [wt/vol] glucose) at 32°C on modified BG11 medium (52) with continuous illumination by cool white fluorescent light in an environmental incubator. Aeration during growth was achieved by growing 500 ml cultures in 2-liter Erlenmeyer flasks with shaking at 140 strokes per min. Growth was measured by the increase in turbidity at 730 nm, where 1 A\textsubscript{730} unit is equal to 0.25 \times 10\textsuperscript{8} cells per ml. For the experiments in which total tRNA was isolated, cultures of \textit{Synechocystis} 6803 were grown under the following conditions: photoautotrophy (BG11 minimal medium plus light); photobacteriotherapy (BG11 minimal medium plus light plus 0.2\% [wt/vol] glucose); photobacteriotherapy with either 12 or 24 h of darkness prior to cell harvesting; photobacteriothermically with ALA (5 and 50 \mu M); photobacteriothermically plus hemin (5 and 25 \mu M); and photobacteriothermically plus levulinic acid (100 \mu M/ml). Cultures (500 ml) containing supplements were inoculated with 10 ml of a saturated culture of \textit{Synechocystis} 6803 and grown to an A\textsubscript{730} = 1.0 in 40 h.

Extraction and determination of chlorophyll \textit{a}. Chlorophyll \textit{a} concentrations were determined as published before (7) from 30-ml portions of \textit{Synechocystis} 6803 cultures. The cells were pelleted by centrifugation and then extracted in dim light with three 5-ml portions of methanol. The methanol extracts were pooled, and the chlorophyll \textit{a} content was measured by determining the A\textsubscript{665}. The extinction coefficient for chlorophyll \textit{a} in absolute methanol is 74.5 ml/mg-cm at 665 nm (7).

Aminoacyl-tRNA synthetase preparations. Partially purified aminoacyl-tRNA synthetase preparations from \textit{Synechocystis} 6803 and \textit{E. coli} HB101 were prepared from frozen cell pellets that had been suspended in aminoacylation assay buffer (20 mM Tricine-OH [pH 8.0], 10 mM KCl, 5 mM MgCl\textsubscript{2}, 3 mM dithiothreitol). The cell suspension was disrupted by passage through a French pressure cell at 16,000 lb/in\textsuperscript{2}. After addition of phenylmethylsulfonyl fluoride (final concentration, 0.2 mM), the cell homogenate was clarified by centrifugation at 100,000 \times g for 90 min. The supernatant fractions (S-100) were dialyzed against two changes of assay buffer containing 20\% glycerol. Nucleic acids were removed from the S-100 preparation by chromatography on DEAE-cellulose (DE52) pre-equilibrated in assay buffer containing 20\% glycerol. The proteins, recovered by a single 300 mM KCl step elution of the column, were dialyzed against two changes of assay buffer containing 20\% glycerol and stored frozen in small portions at −80°C. Protein concentrations were determined by the Bradford assay (9).

Preparation of tRNA. Total \textit{Synechocystis} 6803 aminoacyl-tRNA was prepared by suspending cell pellets in 0.14 \textit{M} sodium acetate (pH 4.5) containing 1\% sodium dodecyl sulfate (SDS) (1 g [wet weight] of cells per 20 ml of buffer). An equal volume of acidic phenol (prepared by dissolving crystalline phenol in 0.14 \textit{M} sodium acetate [pH 4.5]) was added to the cell suspension and incubated for 30 min at 42°C with vigorous shaking. The aqueous phase was recovered by centrifugation and reextracted once with phenol-chloroform (1:1, vol/vol) and once with an equal volume of chloroform. The nucleic acids were recovered from the aqueous phase by ethanol precipitation. The nucleic acid precipitate was suspended in 0.14 \textit{M} sodium acetate (pH 4.5) and then loaded onto a DEAE-cellulose column pre-equilibrated with the same buffer. The column was washed with 0.14 \textit{M} sodium acetate and then with 0.14 \textit{M} sodium acetate containing 0.3 M NaCl until the A\textsubscript{260} of the effluent reached zero. The

FIG. 1. Routes of ALA formation and metabolic pathways of glutamate acceptor RNA species in \textit{Synechocystis} 6803. CoA, Coenzyme A; Glu, glutamate; 1-semialdehyde.
TABLE 1. Properties of Synechocystis 6803 glutamate-accepting tRNA species

<table>
<thead>
<tr>
<th>tRNA species</th>
<th>Glutamylation by unfraccionated aminoacyl-tRNA synthetase* from:</th>
<th>tRNA^Glu_3-dependant ALA formation*</th>
<th>Substrate for Glu-tRNA^Glu_3-dependant amidotransferase*</th>
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<tr>
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<tr>
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* Data for Synechocystis 6803 are from O'Neill et al. (48). Data for E. coli are from this study. Symbols indicate the ability of fractionated Synechocystis 6803 glutamate-accepting tRNA species to be aminoacylated with [14C]Glu by E. coli GluRS, as described in Materials and Methods.

The tRNA was then eluted with 3 column volumes of 0.14 M sodium acetate (pH 4.5) containing 1.0 M NaCl, followed by the recovery of the RNA by ethanol precipitation. For most experiments the tRNA was decyclated by incubation in 20 mM Tris hydroxide (pH 9.0) for 30 min at 37°C. The yield of tRNA was approximately 1 mg of tRNA per g (wet weight) of cells.

The fractionation of Synechocystis tRNA^Glu_3 species by RPC-5 reversed-phase chromatography, performed as described previously (48), yielded four species of glutamate-accepting RNA, and their properties are summarized in Table 1. Two of these tRNAs (tRNA^Glu_3 and tRNA^Glu_3) were authentic tRNA^Glu_3 species, as determined by the sequence of their anticodon, and they had approximately equivalent capacities to support in vitro formation of ALA by Synechocystis 6803 cell extracts (48). The two other glutamate-accepting species (tRNA^Glu_3 and tRNA^Glu_3) were identified as tRNA^Glu_3 species mischarged with glutamate, which should act as a substrate in the tRNA-dependent production of Gln-tRNA^Glu_3 (32) (see also bottom of Fig. 1) and by their inability to be aminoacylated with [14C]Glu by E. coli GluRS (Table 1). As discussed below, estimates of the glutamate-accepting activity in unfraccionated tRNA samples had to be corrected for the occurrence of mischarged Glu-tRNA^Glu_3.

Aminoacylation assays. Aminoacylation assays were performed in a 50-μl reaction mix containing 20 mM Tricine-Cl (pH 8.0), 10 mM KCl, 5 mM MgCl₂, 20% glycerol, 3 mM dithiothreitol, 2 mM ATP, 0.1 to 2.0 A₂₆₀ units of tRNA, and the appropriate 14C-labeled amino acid at a final concentration of 20 to 50 μM. The reactions were started by the addition of 5 to 30 μg of protein of the aminoacyl-tRNA synthetase preparation. After incubation for 15 min at 37°C, the reactions were stopped by spotting the mixtures onto filter paper disks, which were then washed twice for 15 min in 10% trichloroacetic acid, twice for 15 min in 5% trichloroacetic acid, and once for 10 min in ice-cold ethanol. The filters were dried, and the radioactivity was determined by scintillation counting. As noted above, Synechocystis 6803 GluRS mischarges tRNA^Glu_3 with Glu (57). In order to estimate the fraction of Glu-tRNA^Glu_3 versus Glu-tRNA^Glu_3 and Gln-tRNA^Glu_3, samples of Synechocystis 6803 tRNA which had been charged with [14C]Glu were extracted with acidic phenol, and the charged tRNAs were separated from free glutamate by three ethanol precipitations. The charged tRNAs were deacylated by suspension in 100 μl of aminoacylation assay buffer, mixed with 0.4 ml of 20 mM KOH, and incubated at 24°C for 2 min. The mixture was neutralized by the addition of 81 μl of 0.1 M HCl, passed over a small column (0.2 ml) of Dowex-1 resin (preequilibrated with water), and then washed with 3 column volumes of water. At neutral pH, glutamate is bound by the Dowex-1 resin, while glutamine is contained in the flowthrough fraction. The bound glutamate was eluted from the column with 3 column volumes of 0.1 M HCl. The radioactivity in the flowthrough (Gln) and bound (Glu) fractions was determined by scintillation counting. By this method, we determined that in aminoacylation assays (in the presence of an amide donor such as glutamine) in which a Synechocystis 6803 aminoacyl-tRNA synthetase is used to charge Synechocystis 6803 crude tRNA, approximately 80% of the radioactivity bound to tRNA is glutamate. This method was used to estimate the levels of tRNA^Glu_3 in crude tRNA populations.

DNA isolation. Genomic DNA was isolated from Synechocystis 6803 cells as described before (17). The method involved suspension of the cells in buffer containing 50 mM Tris chloride (pH 7.8), 40 mM EDTA, and 1% SDS, grinding the cells with a mortar and pestle in the presence of liquid nitrogen, and three extractions of the cell homogenate with phenol. The nucleic acids were recovered from the aqueous phase by ethanol precipitation and further purified by centrifugation through a CsCl density gradient containing ethidium bromide (40). Plasmid DNA from E. coli was prepared by the alkaline extraction method (40).

Oligonucleotide synthesis and labeling. Two oligonucleotides, based on the Synechocystis 6803 tRNA^Gln sequence (48), were synthesized for these studies. Oligonucleotide I214 (5'-GCCCCCATCGTCTAGAGGCC-3') is identical to nucleotides 1 to 20 of the tRNA^Gln sequence (numbering according to reference 60). Oligonucleotide CI07 (5'-GGCCTCTAGAGCATGGGGGC-3') is complementary to nucleotides 1 to 20 of tRNA^Glu_3. They were phosphorylated at their 5' end by using T4 polynucleotide kinase and [γ-32P]ATP (40).

Southern blot analysis. Restriction fragments obtained by digestion of Synechocystis 6803 chromosomal DNA were separated by electrophoresis through 0.7% agarose gels and transferred to nitrocellulose sheets (40). The blots were screened with the 32P-labeled I214 oligonucleotide. The nitrocellulose filters were hybridized for 18 h at 42°C in 5× SSC (0.75 M NaCl, 0.75 M sodium citrate [pH 7.0]-2× Denhardt solution (0.04% bovine serum albumin, 0.04% Ficoll, 0.04% polyvinylpyrrolidone)-0.1% SDS—50 μg of denatured calf thymus DNA per ml. In order to remove nonspecifically bound probe, the nitrocellulose sheets were washed in 2× SSC containing 0.1% SDS three times for 20 min each at 23°C and twice at 48°C for 30 min each. Hybridization was detected by autoradiography.

Northern (RNA) blot analysis. Synechocystis 6803 tRNA was fractionated by electrophoresis through 10% polyacrylamide-8 M urea gels and electroblotted to Zeta Probe nylon membranes (Bio-Rad) as described by the manufacturer with 20 mM Tris (pH 7.2)-2.5 mM sodium acetate—1 mM disodium EDTA as the transfer buffer. Northern blot hybridizations were performed at 50°C in a mixture containing 5× SSC, 50 mM NaH₂PO₄, 2× Denhardt solution, 100 μg of salmon sperm DNA per ml, and 0.2% SDS. The C107 oligonucleotide was used in the Northern blot hybridization for 18 h, the blots were washed three times for 30 min each at 52°C in 2× SSC-0.2% SDS and analyzed by autoradiography.

Cloning of the Synechocystis 6803 tRNA^Glu_3 gene. Synechocystis 6803 DNA was digested to completion with HindIII and BglII, and the restriction fragments were sepa-
rated by electrophoresis on a 0.8% agarose gel. DNA fragments comigrating with a 4.3-kb marker were eluted from the gel by using DEAE-cellulose paper (Schleicher & Schuell), and the recovered DNA fragments were ligated into the HindIII and BamHI sites of the vector pUC19 (64). The ligation reaction mix was transformed into *E. coli* XL-1 by electroporation (16), and the clones containing recombinant plasmids were transferred to nitrocellulose sheets and screened by colony hybridization with the 32P-labeled oligonucleotide I214. Hybridizations were for 4 h at 42°C in 5× SSC-2× Denhardt solution-0.1% SDS—50 μg of denatured calf thymus DNA per ml. In order to remove nonspecifically bound probe, the colony blots were washed in 2× SSC—0.1% SDS three times for 20 min each at 23°C and twice at 48°C for 30 min each. Positive colonies were detected by autoradiography for 18 h.

**DNA sequencing.** Both M13 single-stranded phage and double-stranded, supercoiled plasmid DNAs were sequenced by the dideoxy chain-termination method (55) with [α-32P]dATP and the Sequenase sequencing kit (United States Biochemical Corp., Cleveland, Ohio). Subclones of the 4.3-kb *HindIII-BglII* *Synechocystis* 6803 DNA fragment containing the tRNA<sub>Glu</sub> gene were obtained by using (i) the *XbaI* and the *EcoRI* restriction endonuclease sites which occur in the sequences specifying the dihydrouridine loop and the T-pseudouridine loops, respectively, of the tRNA<sub>Glu</sub> gene; (ii) two *DraI* restriction endonuclease sites occurring 37 nucleotides to the 5' side and 76 nucleotides to the 3' side of the tRNA<sub>Glu</sub> gene; and (iii) a second *EcoRI* site that is located between the *EcoRI* site in the tRNA<sub>Glu</sub> gene and the *BglII* site. The two oligonucleotides, I214 and CI07, described above were also used as DNA sequencing primers to obtain sequence data. Nucleotide sequence analysis was performed with the University of Wisconsin Genetics Computer Group programs (15) and release 62 of the GenBank data base.

**Assay for in vitro protein synthesis with a coupled transcription-translation system.** Based on earlier work (54), an in vitro transcription-translation system completely dependent on added, precharged Glu-tRNA<sub>Glu</sub> was prepared from *E. coli* JP1449, which has a thermolabile GluRS (37, 53). The programming template (plasmid pBSK::DHFR) was constructed using the *E. coli* dihydrolipoamide S-malyltransferase gene (44), which was obtained on a 1.057-kb *HindIII* fragment (44), downstream of a 7.7-kb T7 RNA polymerase-dependent promoter in the *HindIII* site of the plasmid pBSK (Stratagene). The dhfr mRNA transcripts were generated by preincubation of the programming plasmid in the presence of purified T7 RNA polymerase. The S30 extract was prepared as described by Nirenberg (43) with the modifications of Chen and Zubay (13) from 8 g (wet weight) of *E. coli* JP1449 cells (grown at 28°C in Luria broth). The S30 extract was incubated for 60 min at 30°C to allow the endogenous mRNA to be degraded. At 30°C the specific activity of GluRS in the S30 extract was approximately 25% of that found in other *E. coli* strains, such as HB101 and DP253. There was no detectable GluRS activity in S30 extracts of JP1449 that had been incubated at 40°C for 5 min. The composition and final concentrations of components in the 0.3-ml transcription-translation reaction mixtures were 44 mM Tris-acetate (pH 8.2), 27 mM ammonium acetate, 55 mM potassium acetate, 7.5 mM calcium acetate, 15 mM magnesium acetate, 1.4 mM diithiothreitol, 0.22 mM each of 19 unlabeled amino acids (no glutamate), 2.2 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.5 mM GTP, 20 mM sodium phosphoenol pyruvate (pH 7), 27 μg of pyridoxine hydrochloride per ml, 27 μg of NADP per ml, 27 μg of flavin adenine dinucleotide per ml, FAD, 27 μg of folic acid per ml, 4 A<sub>260</sub> units of *E. coli* MRE600 crude tRNA, DNA (15 μg of the programming plasmid pBSK::DHFR), and 0.5 U of purified T7 RNA polymerase. The components of the transcription-translation reaction mix were mixed and incubated for 5 min at 30°C. The S30 extracts were separately incubated at 30 or at 40°C to inactivate the GluRS. Translation reactions were initiated by mixing the buffer with 0.5 mg of S30 extract protein and, where indicated, with either 3 μCi of [14C]glutamate (283 mCi/mmol) or 100 pmol (40,500 cpm) of precharged [1-14C]Glu-tRNA. The reaction mixtures were incubated for 1 h at 30 or 40°C, and the reactions were stopped by the addition of 1.0 ml of 5% (wt/vol) trichloroacetic acid. The samples were incubated at 95°C for 20 min in order to hydrolyze any aminocyl-tRNA and chilled at 0°C for 20 min, and then the precipitate was collected on glass fiber filter disks (Whatman) by vacuum filtration. Each filter was washed three times with 10 ml of 5% (wt/vol) trichloroacetic acid and once with 10 ml of ethanol. The filters were dried, and the radioactivity was determined by scintillation counting.

**Nucleotide sequence accession number.** The tRNA<sub>Glu</sub> gene sequence has been assigned GenBank accession no. M32099.

**RESULTS**

*Synechocystis* 6803 chromosomal DNA contains a single tRNA<sub>Glu</sub> gene. We first wanted to analyze the organization of tRNA<sub>Glu</sub> genes in *Synechocystis* 6803. As the two tRNA<sub>Glu</sub> species from this organism had identical primary nucleotide sequences and differed only in the modification of the first anticodon base, 5-methylaminomethyl-2-thiouridine (48), we synthesized an ekioskadeoxynucleotide (identical to the 5'-terminal sequence of the tRNA) as a hybridization probe for Southern blot analysis. This sequence was also chosen because it was dissimilar to other known tRNAs; therefore, nonspecific cross-hybridization should be minimized. Only a single hybridizing DNA fragment could be detected by Southern blotting in all single and double restriction endonuclease digests that were analyzed, indicating that a single copy of the tRNA<sub>Glu</sub> gene is present in the *Synechocystis* 6803 genome (Fig. 2). This gene was contained in a 4.3-kb *HindIII-BglII* DNA fragment (Fig. 2), which was isolated by cloning into the *HindIII-BamHI* sites of pUC19 to yield plasmid pS6TE.

**Sequence of the tRNA<sub>Glu</sub> gene.** The location of the tRNA<sub>Glu</sub> gene within the 4.3-kb fragment was determined by mapping the positions of *XbaI* and *EcoRI* restriction sites. The nucleotide sequence of the tRNA<sub>Glu</sub> (48) predicts that these sites occur in the dihydrouridine and T-pseudouridine loops. The DNA sequence of a 1,000-bp region around the tRNA<sub>Glu</sub> gene was determined; the pertinent sequences are shown in Fig. 3. The complete sequence has been deposited with GenBank (accession number M32099). The coding region is identical to the previously determined tRNA<sub>Glu</sub> sequence (48) except that the -CCA end of the mature tRNA is not encoded in the gene. Computer analysis revealed putative promoter sequences and also a stringent response element (20, 65) in the 5' flank (Fig. 3). A rho-independent transcription terminator sequence in the 3' flank of the tRNA<sub>Glu</sub> gene was identified by the method of Brendel and Trifonov (10). Sequences capable of forming significant stem-loop secondary structures were found in both the 5' and 3'-flanking sequences (Fig. 3). Within the sequenced region, no other tRNA, rRNA, or protein-coding genes could be detected.
SYNECHOCYSTIS SP. STRAIN PCC 6803 tRNA\textsuperscript{Glu} GENE

FIG. 2. Southern hybridization of a Synechocystis 6803 tRNA\textsuperscript{Glu}-specific 32P-oligonucleotide with Synechocystis 6803 genomic DNA. Restricted DNA (5 \(\mu\)g) was separated on a 0.7% agarose gel and transferred to nitrocellulose paper (40). Hybridization of the 32P-labeled oligonucleotide 1214 was performed as described in Materials and Methods. The lanes contained Synechocystis 6803 genomic DNA digested with HindIII (lane 1), HindIII and KpnI (lane 2), KpnI (lane 3), BglII (lane 4), and HindIII and BglII (lane 5). Fragment sizes of HindIII-cleaved lambda DNA are shown in kilobases (kb).

The putative transcriptional control elements and the lack of other identifiable genes suggest that the tRNA\textsuperscript{Glu} gene is expressed as a monocistron transcriptional unit. Northern blot analysis of tRNA preparations from E. coli cells transformed with plasmid pS6TE showed that the Synechocystis 6803 tRNA\textsuperscript{Glu} gene was transcribed and processed to form mature-sized tRNA\textsuperscript{Glu} in E. coli (data not shown). However, we do not know whether E. coli accurately processes the 3' end of this tRNA.

Synechocystis 6803 contains a functional excess of Glu-tRNA\textsuperscript{Glu} under different growth conditions. As Synechocystis 6803 uses Glu-tRNA\textsuperscript{Glu} for porphyrin synthesis as well as for protein biosynthesis we wanted to determine whether the demand for this tRNA during photosynthetic growth is satisfied by normal constitutive expression of its gene or whether expression is upregulated. In order to test this, total tRNA was isolated from Synechocystis 6803 cultures grown under a variety of conditions which lead to greatly varied chlorophyll levels. The culture conditions tested included photoautotrophic growth, photoheterotrophic growth in the presence of glucose, 24-h darkening of the cultures prior to harvesting, and the addition to the cultures of known inhibitors of ALA or chlorophyll biosynthesis, such as hemin (30), ALA (58), gabaculinc (29, 48), and levulinic acid (4). The levels of chlorophyll \(a\) in the various cultures varied over a 10-fold range from 0.002 pg (in gabaculin-treated cells) to 0.024 pg of chlorophyll \(a\) per cell (in photoheterotrophically grown cells) (Fig. 4C). This figure also shows that the relative tRNA\textsuperscript{Glu} levels in the unfraccionated tRNA samples remained constant, as determined by Northern blot analysis with a specific oligonucleotide. Aminoacylation assays confirmed these data (Table 2). The amount of unfraccionated tRNA also remained approximately the same under these conditions (Table 2). Table 2 summarizes the representative data for the cultures which showed the greatest variation in chlorophyll content. As the Synechocystis 6803 GluRS, like that of other gram-positive bacteria (57), glutamylates tRNA\textsuperscript{Glu} in addition to tRNA\textsuperscript{Glu} (see Materials and Methods and Fig. 1 legend for detailed explanation), an additional experiment was needed to determine the amounts of Glu-tRNA\textsuperscript{Glu} needed. This was done by charging total Synechocystis cellular tRNA with E. coli GluRS, which is unable to glutamylate tRNA\textsuperscript{Glu}. As shown in Table 2, the levels of tRNA\textsuperscript{Glu} did not change. The acceptor activities for other amino acids did not vary significantly in the different tRNA preparations; the amino acid acceptance of unfraccionated tRNA was 54, 27, 40, 28, 70, 29, 49, and 35 pmol/A\textsubscript{260} unit. Northern blot analysis performed as (lane 1), HindIII and KpnI (lane 2), KpnI (lane 3), BglII (lane 4), and HindIII and BglII (lane 5). Fragment sizes of HindIII-cleaved lambda DNA are shown in kilobases (kb).

FIG. 3. Nucleotide sequence of the Synechocystis 6803 tRNA\textsuperscript{Glu} gene. The tRNA\textsuperscript{Glu} sequence is presented in lowercase letters. Putative promoter elements (~35 region [TTGACA] and ~10 region [TATATT]) and a sequence possibly involved in the stringent response (GTCTGA) are indicated by asterisks. Two sequences capable of forming stem-loop structures are underlined. The GenBank accession number for this sequence is M32099.
Expression of GluRS. Although expression of tRNA\textsuperscript{Glu} does not appear to be coregulated with chlorophyll biosynthesis, we considered that the expression of GluRS, the cognate aminoacyl-tRNA synthetase, may be regulated. It is well known that aminoacyl-tRNA synthetases are regulated by a variety of mechanisms at the transcriptional, translational, and physiologic levels (25). Therefore, in a parallel experiment to the studies described above, partially purified GluRS fractions were prepared from cultures of Synechocystis 6803 grown under the same conditions which provided the cells for tRNA and chlorophyll a determinations. The relative specific activity of GluRS was twofold higher in extracts from photoheterotrophically grown cultures than in extracts that had been prepared from cultures which had been darkened for 24 h prior to harvesting (Table 2). However, GluRS activity was not related to chlorophyll concentration (Table 2). Substantial decreases in GluRS activity were observed in cultures that had been incubated in the dark or treated with gabaculin. This effect is most likely the result of the metabolic regulation of GluRS in Synechocystis 6803. Metabolic regulation, which affects all E. coli aminoacyl-tRNAs synthetases examined to date, is a growth rate-limited control mechanism; a two- to threefold increase in synthetase level accompanies a fivefold increase in the growth rate (25). In our studies, the substantially lower growth rates in the darkened and gabaculin-treated cultures (21 and 26\%), respectively, of that of photoheterotrophically grown cells) most likely led to a general reduction in aminoacyl-tRNA synthetase expression.

**Both Synechocystis 6803 tRNA\textsuperscript{Glu} species can participate in protein biosynthesis.** As there is only one tRNA\textsuperscript{Glu} gene encoded in the Synechocystis 6803 genome, the same gene must provide tRNA that functions in both protein and ALA biosynthesis. As two tRNA species are formed (48), it is possible that differences in tRNA modification may lead to preferential use of one of the species for protein or ALA biosynthesis. To test this, we examined the ability of the two purified, precharged Synechocystis 6803 tRNA\textsuperscript{Glu} species to provide glutamate for protein synthesis in an in vitro transcription-translation system. For this experiment we chose the heterologous E. coli system (i) because of the ability to make this extract totally dependent on added, precharged Glu-tRNA and to use a defined mRNA and (ii) to circumvent the complications resulting from the ability of the Synechocystis 6803 GluRS to charge tRNA\textsuperscript{Glu} in addition to tRNA\textsuperscript{GGU} (57). These criteria cannot be met with the crude homologous Synechocystis 6803 system used earlier (56) (see Discussion). In order to eliminate the formation of Glu-tRNA during the experiment, the cell extract was prepared from E. coli JP1449, which contains a thermolabile GluRS as a consequence of the gltX mutation (37, 53). After incubation of this extract at 40°C, glutamate incorporation into proteins depends solely on added Glu-tRNA. Thus, the two Synechocystis 6803 Glu-tRNA\textsuperscript{Glu} species can be tested for their ability to function in protein biosynthesis. The cell extract was programmed with the E. coli dhfr gene (see Materials and Methods). The DHFR protein has 12 glutamate residues in its 159-amino-acid sequence (59). As shown in Table 3, there was only negligible incorporation in the absence of added tRNA. Protein synthesis proceeded well in the presence of tRNA and glutamate at 30°C, but at 40°C the incorporation was reduced to 2% of that observed at the lower temperature. This demonstrates that heat inactivation of the thermolabile mutant GluRS eliminates the protein-synthetic ability of the extract. At the nonpermissive temperature of 40°C and at 30°C, precharged

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**FIG. 4.** Northern blot hybridization showing levels of tRNA\textsuperscript{Glu} in RNA preparations and chlorophyll content from cultures of Synechocystis 6803. (A) Ethidium bromide-stained gel (10% polyacrylamide–8 M urea) of total RNA from various cultures of Synechocystis 6803. RNA was isolated and fractionated by electrophoresis (see Materials and Methods) and stained with ethidium bromide (1 \(\mu\)g/ml). Lanes 1 to 12. Total low-molecular-weight RNA from Synechocystis 6803 cultures grown as follows: photoheterotrophically (BG11 minimal medium plus light plus glucose plus levulinic acid) (lane 1); photoheterotrophically plus gabaculin (10 \(\mu\)M [lane 2] and 1 \(\mu\)M [lane 3]); photoheterotrophically plus hemin (25 \(\mu\)g/ml [lane 4] and 5 \(\mu\)g/ml [lane 5]); photoheterotrophically plus ALA (50 \(\mu\)g/ml [lane 6] and 5 \(\mu\)g/ml [lane 7]); photoheterotrophically with either a 24-h (lane 8) or a 12-h (lane 9) period of darkness prior to harvesting; photoheterotrophically with 0.2% glucose (lane 10) and 0.02% glucose (lane 11); and photouautotrophically (BG11 minimal medium plus light) (lane 12). Lane 13, HindIII-cleaved pBR322 (denatured). The positions of standards (numbers indicate bases) are indicated at the right. Each lane contains 10 \(\mu\)g of total RNA. The tRNA\textsuperscript{Glu} comigrated with the 75-base marker. (B) Autoradiogram obtained after Northern transfer of RNA gel described for panel A. Following electrophoresis, the RNA was transferred to a nylon membrane and then hybridized with the \(^{32}P\)-labeled tRNA\textsuperscript{Glu}, specific oligonucleotide C107. The lanes in panel B are aligned with the equivalent lanes of panel A. (C) Chlorophyll content in the various cultures. The chlorophyll content of a portion of cells from each culture was determined and is expressed as femtomoles of chlorophyll a per cell. The values for chlorophyll content are aligned with the corresponding lanes in panels A and B.
crude *Synechocystis* [14C]Glu-tRNA, purified [14C]Glu-tRNA<sub>Glu</sub>, and purified [14C]Glu-tRNA<sub>Glu</sub> were all capable of donating glutamate for protein synthesis (Table 3). No significant difference was observed when the two tRNA<sub>Glu</sub> species were compared. As expected, the addition of presterilized *E. coli* Glu-tRNA to the transcription-translation reactions mixes incubated at 40°C resulted in the transfer of Glu into protein. These experiments indicate that the two *Synechocystis* 6803 tRNA<sub>Glu</sub> species can, at least in an *E. coli* system, function in protein biosynthesis.

**DISCUSSION**

*Synechocystis* 6803 tRNA<sub>Glu</sub> gene. Modern methods of molecular biology have uncovered the arrangement of tRNA genes in the genomes of various organisms (1, 34). The number and organization of tRNA genes vary in different organisms. The monocistronic organization of the *Synechocystis* 6803 tRNA<sub>Glu</sub> gene is dissimilar to that of tRNA<sub>Glu</sub> genes in *E. coli* and *Bacillus subtilis* (34, 65). In *E. coli*, copies of the tRNA<sub>Glu</sub> genes are contained in several rRNA operon genes encoding 16S and 23S rRNAs (20). In *B. subtilis*, tRNA<sub>Glu</sub> genes are found in multimeric operons which code for up to 20 tRNAs (65). To date, only two cyanobacterial tRNA genes (for tRNA<sub>Ala</sub> and tRNA<sub>Phe</sub>) in *Anacystis nidulans* have been cloned, and their location in the spacer region between the 16S and 23S rRNA genes resembles the arrangement of the corresponding genes in *E. coli* and *B. subtilis* (63, 66). *trnE* genes have been isolated from cyanobacteria and from algal and higher-plant chloroplasts; in several cases the *trnE* gene was shown to be cotranscribed with other tRNAs (8, 15, 27, 28, 36, 45, 46, 49, 50). As in the case of *Synechocystis* 6803, the *trnE* gene is present in a single copy in the genomes of cyanobacteria and of algal and higher-plant chloroplasts.

Although a detailed analysis of transcription initiation and termination in cyanobacteria is not available, sequence analysis of cloned cyanobacterial genes (62, 67) indicates that transcriptional control elements in cyanobacteria are similar to those in *E. coli* and *B. subtilis* (41). The putative transcriptional signals of the *Synechocystis* 6803 tRNA<sub>Glu</sub> gene are nearly identical to the characteristic –35, –10, and stringent-response sequences found in *B. subtilis* tRNA genes (65). Interestingly, the *Synechocystis* 6803 –35 site is located in a sequence that is capable of forming a stable stem-loop structure. Such a structure may be required for interaction with a regulatory protein (41), while the stem-loop structure in the 3' end of the gene is characteristic of rho-independent procaryotic terminators (20, 41, 65).

**Chlorophyll and tRNA<sub>Glu</sub> biosynthesis are not coregulated.** The aim of this study was to investigate possible ways that *Synechocystis* uses to cope with the demand for tRNA<sub>Glu</sub> for protein biosynthesis and for ALA formation. We chose to search for coregulation with chlorophyll biosynthesis because it has been suggested that there is feedback inhibition of ALA formation by chlorophyll precursors (reviewed in reference 6). Although in cyanobacteria the major tetrapyrole end product is phycocyanobilin (6), there is no reported link between the regulation of phycocyanobilin synthesis and ALA formation in these organisms. As a matter of fact, phycocyanobilins and chlorophyll are biosynthesized by different routes; the synthesis of phycocyanobilins follows the same route as for hemes. The biosynthesis of the two latter molecules is probably regulated independently from that of chlorophyll (6).

We have shown that both *Synechocystis* 6803 tRNA<sub>Glu</sub> species involved in ALA biosynthesis also function in protein biosynthesis. This conclusion was reached in an experiment with the heterologous *E. coli* system. Although a homologous system had been used before for this purpose, we decided to use the *E. coli* system because of deficiencies of the homologous system (56). In contrast to the *Synechocystis* cell extract, *E. coli* preparations could be made completely dependent upon added charged Glu-tRNA (by using an extract from a strain carrying a temperature-sensitive GluRS; see Materials and Methods) and programmed with a defined mRNA template. In addition, we

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**TABLE 2. Levels of glutamate acceptor RNA and GluRS activity under various growth conditions**

<table>
<thead>
<tr>
<th>Growth conditionsa</th>
<th>Chlorophyll content (fg/cell)</th>
<th>Total RNA (fg/cell)</th>
<th>Total amount (pmol/30,000 unit of tRNA)</th>
<th>Fraction of Glu-tRNA&lt;sub&gt;Glu&lt;/sub&gt; aminoacylated in vivo (%)</th>
<th>GluRS activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light + glucose</td>
<td>24</td>
<td>5.2</td>
<td>43.6</td>
<td>34.9</td>
<td>99.5</td>
</tr>
<tr>
<td>Light + glucose + 24-h darkening</td>
<td>20</td>
<td>4.8</td>
<td>37.4</td>
<td>32.0</td>
<td>99.2</td>
</tr>
<tr>
<td>Light + glucose + 10 μM gabaculin</td>
<td>2</td>
<td>4.3</td>
<td>36.6</td>
<td>29.3</td>
<td>99.6</td>
</tr>
</tbody>
</table>

a For growth conditions, see Materials and Methods. Cultures were grown to an A<sub>680</sub> of ≈1.0.

b Aminoacyl-tRNA was extracted and purified at pH 4.5 as described in Materials and Methods. One-half of the sample was dec酰lated by incubation at pH 9.

c Both aminoacylated and deacylated tRNA samples were assayed for glutamate acceptance. Total Glu acceptor tRNA (Glu-tRNA<sub>Glu</sub> and Glu-tRNA<sub>Glu</sub>) was determined with *Synechocystis* 6803 GluRS. Total tRNA<sub>Glu</sub> was determined by glumylation with *E. coli* GluRS.

c Aminoacyl-tRNA synthetase extracts were prepared as described in Materials and Methods; 5 μg was used per assay. One unit of GluRS activity is equal to the amount of protein which forms 1 pmol of Glu-tRNA during 15 min of incubation at 37°C.

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**TABLE 3. In vitro protein synthesis supported by various [14C]Glu-tRNA species**

<table>
<thead>
<tr>
<th>Amendments</th>
<th>Glutamate incorporation (pmol)</th>
<th>30°C</th>
<th>40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>No tRNA, + [14C]Glu</td>
<td>0.9</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>+ <em>Synechocystis</em> crude tRNA, + [14C]Glu&lt;sub&gt;a&lt;/sub&gt;</td>
<td>69.2</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>+ <em>Synechocystis</em> 6803 crude [14C]Glu-tRNA</td>
<td>18.3</td>
<td>18.3</td>
<td></td>
</tr>
<tr>
<td>+ <em>Synechocystis</em> 6803 [14C]Glu-tRNA&lt;sub&gt;Glu&lt;/sub&gt;</td>
<td>22.3</td>
<td>20.4</td>
<td></td>
</tr>
<tr>
<td>+ <em>Synechocystis</em> 6803 [14C]Glu-tRNA&lt;sub&gt;Glu&lt;/sub&gt;</td>
<td>18.0</td>
<td>17.0</td>
<td></td>
</tr>
<tr>
<td>+ <em>E. coli</em> [14C]Glu-tRNA&lt;sub&gt;Glu&lt;/sub&gt;</td>
<td>24.3</td>
<td>28.4</td>
<td></td>
</tr>
</tbody>
</table>

a Experimental details are described in Materials and Methods. Reaction mixes that did not contain free [14C]glutamate were supplemented with cold glutamate to give a final concentration of 1 mM.

b This incubation mix contained only [14C]glutamate which was charged by GluRS in the extract during the protein synthesis reaction. Therefore, higher incorporation was expected compared with protein synthesis from precharged [14C]Glu-tRNA, for which incorporation of deacylated tRNA was not measured because of a 2,000-fold excess of unlabeled glutamate in those reaction mixes.

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SYNECOCHYSITIS SP. STRAIN PCC 6803 tRNA<sub>Glu</sub> GENE

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used purified *Synechocystis* tRNA<sub>Glu</sub> which had been separated from tRNA<sup>Gln</sup> by chromatography and electrophoresis (see Materials and Methods); in this way we excluded glutamylation of tRNA<sub>Glu</sub> (57). Thus, it is clear that the two *Synechocystis* tRNA<sub>Glu</sub> species participate in protein synthesis in *E. coli*. This probably also pertains to the homologous system, although it is possible that *Synechocystis* 6803 contains additional factors which may affect this process. However, this does not appear to occur, since both tRNA species apparently function equally well in a homologous translation system (56).

Our data show that there is no gene amplification, no increase in the tRNA<sub>Glu</sub> level, and no change in the fraction of in vivo charged tRNA<sub>Glu</sub> when chlorophyll synthesis is maximal during photosynthetic growth of *Synechocystis* 6803. These results suggest that chlorophyll and tRNA<sub>Glu</sub> biosynthesis are not coregulated and that a functional excess of Glu-tRNA<sub>Glu</sub> provides the cell with sufficient activated glutamate for both protein and ALA biosynthesis.

**How is tRNA<sub>Glu</sub> routed for ALA or for protein biosynthesis?** How tRNA<sub>Glu</sub> is routed remains an open question. Possibly the relative binding constants for the tRNA<sub>Glu</sub> species of elongation factor EF-Tu and of Glu-tRNA reductase may be important. These proteins will form the first complex with tRNA<sub>Glu</sub> during the process of protein or ALA synthesis. The extent of tRNA modification may also play a role. Additional factors may also be required for routing the tRNAs into separate metabolic pathways.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


