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Transcription of Glutamine Synthetase Genes (*glnA* and *glnN*) from the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 Is Differently Regulated in Response to Nitrogen Availability

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In the cyanobacterium *Synechocystis* sp. strain PCC 6803 we have previously reported the presence of two different proteins with glutamine synthetase activity: GSI, encoded by the *glnA* gene, and GSIII, encoded by the *glnN* gene. In this work we show that expression of both the *glnA* and *glnN* genes is subjected to transcriptional regulation in response to changes in nitrogen availability. Northern blot experiments and transcriptional fusions demonstrated that the *glnA* gene is highly transcribed in nitrate- or ammonium-grown cells and exhibits two- to fourfold-higher expression in nitrogen-starved cells. In contrast, the *glnN* gene is highly expressed only under nitrogen deficiency. Half-lives of both mRNAs, calculated after addition of rifampin or ammonium to nitrogen-starved cells, were not significantly different (2.5 or 3.4 min, respectively, for *glnA* mRNA; 1.9 or 1.4 min, respectively, for *glnN* mRNA), suggesting that changes in transcript stability are not involved in the regulation of the expression of both genes. Deletions of the *glnA* and *glnN* upstream regions were used to delimit the promoter and the regulatory sequences of both genes. Primer extension analysis showed that structure of the *glnA* gene promoter resembles those of the NtcA-regulated promoters. In addition, mobility shift assays demonstrated that purified, *Escherichia coli*-expressed *Synechocystis* NtcA protein binds to the promoter of the *glnA* gene. Primer extension also revealed the existence of a sequence related to the NtcA binding site upstream from the *glnN* promoter. However, *E. coli*-expressed NtcA failed to bind to this site. These findings suggest that an additional modification of NtcA or an additional factor is required for the regulation of *glnN* gene expression.

Glutamine synthetase (GS) catalyzes the ATP-dependent synthesis of glutamine from ammonium and glutamate (49). Glutamine is utilized not only for protein synthesis but also for the synthesis of a number of nitrogen-containing metabolites such as purines, pyrimidines, and amino sugars (38). In addition, in many microorganisms (including cyanobacteria) assimilation of ammonium occurs mainly by the sequential action of GS and glutamate synthase (GS-GOGAT pathway) (40). Because of the importance of glutamine in nitrogen metabolism, it is not surprising that both the catalytic activity and the synthesis of GS are finely regulated in many organisms.

Transcriptional regulation of GS-encoding genes has been extensively studied in *Escherichia coli* and other gram-negative bacteria (reviewed in references 25, 33, and 40). In enteric bacteria, the GS structural gene, *glnA*, lies within the operon *glnA-ntrBC*. The *ntrB* and *ntrC* gene products, NR_{II} and NR_I, respectively, are required for the transcriptional regulation of the operon. These proteins are members of the family of two-component bacterial signal transduction systems (50). During nitrogen-limited growth, the DNA binding protein NR_I, in its phosphorylated form, acts as a transcriptional activator, promoting high transcription from the σ^{54}-dependent promoter of the *glnA-ntrBC* operon. Phosphorylation and dephosphorylation of NR_I are catalyzed by the kinase/phosphatase protein NR_{II}, which indirectly senses the nitrogen balance within the cell. The relative pool size of glutamine and 2-oxoglutarate ultimately determines whether NR_{II} will phosphorylate NR_I (22, 33).

Cyanobacteria are photosynthetic prokaryotes that carry out oxygenic photosynthesis like plants. Most cyanobacteria can use nitrate or ammonium ions as a nitrogen source, and some strains are also able to fix dinitrogen (16, 51). Although the existence of NAD- and NADP-dependent glutamate dehydrogenases has been reported in cyanobacteria (9, 15), ammonium is mainly incorporated into carbon skeletons through the GS-GOGAT pathway (27, 28). Expression of the *glnA* gene has been partially characterized in several cyanobacteria. In the unicellular *Synechococcus* sp. strain PCC 7002 the *glnA* transcript level increases three- to fivefold when the cells are nitrogen starved (56). The amount of *glnA* transcript in *Synechococcus* sp. strain PCC 7942 increases strongly after transfer of ammonium-grown cells to nitrate-containing or nitrogen-free medium (11, 24). In the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120, *glnA* is transcribed from multiple promoters that are differentially expressed in response to changes in nitrogen availability (52). In another filamentous strain, *Calothrix* sp. strain PCC 7601, two *glnA* transcripts have been detected. A 1.6-kb mRNA predominates in nitrate-grown cells, while a longer, 1.8-kb species predominates in ammonium-grown cells (13). In addition to nitrogen conditions, other factors may also control the expression of the *glnA* gene. We have recently reported that photosynthetic and respiratory electron transport controls transcription of the *glnA* gene from *Synechocystis* sp. strain PCC 6803 (43).

Regulation of *glnA* gene expression in cyanobacteria appears not to be exerted by an Ntr-like system. The transcriptional activator NtcA, which belongs to the CRP family of bacterial

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DNA binding proteins (18, 53) has been shown to bind directly to the promoter region of *Anabaena* sp. strain PCC 7120 and *Synechococcus* sp. strain PCC 7942 *glnA* genes (24, 39, 58). In addition, NtcA mutants of *Synechococcus* sp. strain PCC 7942 and *Anabaena* sp. strain PCC 7120 are impaired in the expression of the *glnA* gene (17, 24, 54, 59). The structure of the NtcA-regulated promoters has been proposed to be constituted by a −10 *E. coli* consensus box, followed at 22 nucleotides by an NtcA binding site ($GTAN_8TAC$) (24) that substituted for the canonical −35 box.

In the unicellular facultative heterotrophic cyanobacterium *Synechocystis* sp. strain PCC 6803 we have recently reported the existence of two genes encoding two different types of GS: the *glnA* gene, encoding classical prokaryotic dodecameric GS type I (GSI), and the *glnN* gene, encoding a hexameric GS named GS type III (GSIII), similar to the GSs of *Butyrivibrio fibrisolvens* and *Bacteroides fragilis* (19, 41). Sequences homologous to *glnN* were also identified in other non-nitrogen-fixing cyanobacteria. In order to investigate the metabolic role of each GS gene from *Synechocystis* sp. strain PCC 6803, we have studied the regulation of the expression of both genes. In this work we demonstrate that transcription of both genes is regulated in response to changes in nitrogen availability; however, each gene displays a different pattern of expression. Our results point to the *glnN* product as a specific GS for nitrogen deficiency conditions and suggest the existence of specific modifications of NtcA or another transcription factor(s) involved in the regulation of *glnN* gene expression.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *Synechocystis* sp. strain PCC 6803 and its derivatives were grown photoautotrophically at 35°C on BG11 medium (44) (18 mM nitrate as the nitrogen source) under continuous fluorescent illumination (50 $W \cdot m^{-2}$; white light). The cultures were bubbled with 1.5% (vol/vol) CO_2 in air. Alternatively, cultures were grown at 30°C with shaking. $BG11_0$ medium was BG11 medium lacking the nitrogen source. When ammonium was used as the nitrogen source, $BG11_0$ medium was supplemented with 10 mM NH_4Cl and the medium was buffered with 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer. Kanamycin (50 or 200 μg/ml) and chloramphenicol (20 μg/ml) were added when required. *E. coli* DH5α was used as the host for plasmid preparations. *E. coli* MC 1061 was utilized for transformation with plasmid DNA isolated from *Synechocystis*. Luria broth was supplemented with ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, or chloramphenicol at 20 μg/ml when required.

DNA manipulation and DNA sequencing. All DNA manipulations were performed following standard procedures (1, 46). DNA fragments were purified from agarose gels with the GeneClean kit (Bio 101, Inc.). Total DNA from cyanobacteria was isolated as described in reference 4. DNA probes were ^{32}P labelled with a random primer kit (Boehringer Mannheim) using [α-^{32}P]dCTP. Nested deletions of pJCR17 plasmid were performed by using a double-stranded nested deletion kit from Pharmacia. The complete sequence of both strands was determined by the dideoxy chain termination method (47), using Sequenase 2.0 (U.S. Biochemicals).

RNA isolation and Northern blot analysis. Total RNA was isolated from mid-exponential-phase cultures of *Synechocystis* sp. strain PCC 6803 essentially as described by Mohamed and Jansson (34), with the modifications described in reference 43. Separation of RNA on formaldehyde gels, transfer to nylon membranes (Hybond N+; Amersham), and prehybridization and hybridization conditions were according to instruction manuals from Amersham. Fifteen micrograms of total RNA was loaded per lane.

The *glnA* probe was generated by labelling a 631-bp *Eco*RV-*Eco*RV internal *glnA* fragment from pJCR3 (41). The *glnN* probe was generated by labelling a 660-bp *Eco*RI-*Eco*RI *glnN* fragment from pBE2 (41). As a control for sample loading, all the filters were stripped and reprobed with an about 450-bp *Hin*dIII-*Bam*HI fragment from pAV1100 (55) which contains the RNase P RNA gene from *Synechocystis* sp. strain PCC 6803.

Determination of *glnA*- and *glnN*-specific mRNA half-lives. Total cellular RNA from nitrogen-starved *Synechocystis* cells was isolated at several times after addition of rifampin (400 μg/ml) or ammonium (1 mM) to the culture. The decay of *glnA* and *glnN* transcripts was monitored by Northern blot hybridization. After densitometric quantification of the signals on the autoradiograms, the half-life of the transcripts under both conditions was estimated from a semilogarithmic plot of relative transcript level versus time.

Transcriptional gene fusions. Transcriptional gene fusions were constructed in plasmid pFF11, a promoter-probe vector based on the *cat* (chloramphenicol acetyl transferase [CAT]-encoding) gene (14). Different fragments upstream of the *glnA* gene (see Fig. 5 and Table 1) were subcloned in pFF11, yielding plasmids pJCRP1, pJCRP2, pJCRP3, and pJCRP4. In order to generate deletions of the *glnN* upstream region, a *Bss*HII-*Bss*HII polylinker containing fragment of pBluescript II SK(+) was cloned into pJCR15(+), rendering pJCR17. Plasmids containing three nested deletions of the *glnN* 5′ region were named pJCR17.1, pJCR17.2, and pJCR17.3. All resulting plasmids were used to transform *Synechocystis* strain SFCΩ5, harboring the pFCΩ5 plasmid, which is homologous to the incoming vector; this allowed the rescue of the incoming vector by homologous recombination with the resident plasmid (7, 8). To obtain total segregation of recombinant plasmids, *Synechocystis* strain PCC 6803 transformants were cultured with high concentrations of kanamycin (200 μg/ml). Homogeneity of the plasmid population was tested by transforming *E. coli* MC1061 with total DNA from the cyanobacterial transformants and selecting for Sm^r Km^s (pFCΩ5-transformed cells) or Sm^r Km^r (pFF11-derived-plasmid-transformed cells) (14). Plasmids were considered segregated when Sm^r Km^s colonies were not found. For checking the transformation product, plasmids isolated from *E. coli* were analyzed by their restriction patterns.

CAT activity was assayed in vitro at 37°C by the colorimetric procedure (48). CAT specific activity is reported as the number of micromoles of chloramphenicol acetylated per minute per milligram of protein. Crude extracts from *Synechocystis* sp. strain PCC 6803 or *E. coli* cells were prepared with glass beads as described in reference 42, except that the buffer was substituted by 50 mM Tris-HCl, pH 8. Protein in cell extracts was determined by the method of Bradford (3), using ovalbumin as the standard.

Strains SFF16 (14), which contains a promoterless *cat* gene, and SFC57 (7), which contains the *cat* gene under the control of its own promoter, were used as controls.

GS assay. GS activity was determined in situ by using the Mn^{2+}-dependent γ-glutamyltransferase assay in cells permeabilized with mixed alkyltrimethylammonium bromide (30). One unit of GS activity corresponds to the amount of enzyme that catalyzes the synthesis of 1 μmol of γ-glutamylhydroxamate per min.

Protein in whole cells was determined by a modified Lowry procedure (26) using ovalbumin as a standard.

Primer extension analysis. The oligonucleotides used for primer extension were 5′ CGTCTTGGATCCACTTGAGGACTTC 3′ for the *glnA* gene and 5′ CAGCCCACATATCTTCCAGGCGGGA 3′ for the *glnN* gene. Primer extensions were carried out as previously described (36). To determine the size of the extension product, nucleotide sequencing of the appropriate plasmid was carried out by using the same oligonucleotide as a primer.

Gel retardation assays and determination of kinetic parameters. A glutathione *S*-transferase (GST)–NtcA fusion protein was expressed and purified as previously described (36). The indicated fragments were end labeled with [α-^{32}P]dCTP by using Sequenase version 2.0 enzyme. The binding reactions and electrophoresis were carried out as previously described (36). When it was necessary, radioactive bands corresponding to free and bound DNA were quantified in a Molecular Dynamics PhosphorImager.

The equilibrium dissociation constant (K_d) was determined from NtcA saturation experiments, and the data obtained were analyzed by a Hill plot.

RESULTS

Induction of GS activity under nitrogen deficiency. We have previously reported the construction of *glnA*- and *glnN*-disrupted mutants, strains SJCR3 and SJCR6, respectively (41). Figure 1 shows the time course of GS activity after transfer of nitrate-grown cells of wild-type (WT) and mutant strains to nitrogen-free medium. After 12 h of nitrogen deprivation, the GS activity of strains SJCR6 (*glnN* mutant) and SJCR3 (*glnA* mutant) increased 2- and 11-fold respectively, while that of the WT strain increased about 2.5-fold, reaching a level close to the sum of the activities presented by the SJCR6 and SJCR3 mutants. These results show that both GSs (GSI, encoded by the *glnA* gene, and GSIII, encoded by the *glnN* gene) are subject to regulation under nitrogen deficiency conditions.

***glnA* and *glnN* transcript levels depend on nitrogen availability.** Levels of *glnA* and *glnN* transcripts under different nitrogen conditions were determined by Northern blotting. Total RNA was isolated from mid-log-phase *Synechocystis* sp. strain PCC 6803 cells that used nitrate or ammonium as a nitrogen source or after 12 h of nitrogen starvation. As previously reported, the *glnA* transcript was a monocistronic mRNA of 1.6 kb (43) (Fig. 2). The presence of excess 16S rRNA was probably responsible for depleting the signal in the 1.5-kb

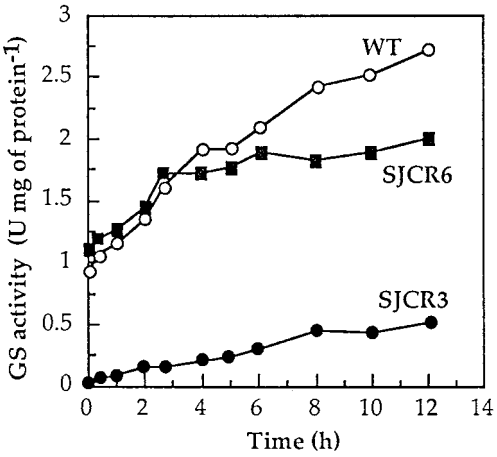


FIG. 1. Time course of GS activity after transfer of nitrate-grown cells to nitrogen-free medium. Mid-log-phase nitrate-grown cells of the WT, SJCR3, and SJCR6 strains were harvested, washed with $BG11_0$ medium (nitrogen-free medium), and resuspended in this medium. At the indicated times, samples were taken for determination of GS transferase activity. Values are averages from three independent experiments.

region (Fig. 2A). Densitometric quantification of the resulting autoradiographs showed that the amount of *glnA* transcript was maximal in nitrogen-deficient cells, being about 40% (of the maximal value) in cells that used nitrate as the nitrogen source and about 25% in ammonium-grown cells (Fig. 2A and B). With a *glnN* fragment as a probe, two bands were detected: a 2.3-kb mRNA that corresponds to the full *glnN* transcript (41) and a 1.2-kb mRNA which is most probably a degradation product of the complete transcript (Fig. 2C). Levels of *glnN* mRNA were extremely low in nitrate- or ammonium-grown cells but detectable after a long film exposition (14 days). The amount of *glnN* transcript increased about 90-fold under nitrogen deficiency (Fig. 2C and D).

Since the maximal level of transcript accumulation for both genes was obtained under nitrogen starvation, we investigated the kinetics of the induction of *glnA* and *glnN* mRNAs after a transfer from nitrate-containing medium to nitrogen-free medium (Fig. 3A and B). The *glnA* and *glnN* relative mRNA levels obtained from two independent experiments were quantified by densitometry, and plots of medium values versus time were drawn (Fig. 3C). The maximal level of transcript accumulation was reached after 2 and 5 to 10 h of nitrogen deprivation for *glnA* and *glnN* transcripts, respectively. Thereafter levels of both transcripts decreased. In fact, after 20 h, the amount of *glnA* mRNA was similar to that present at the beginning of the experiment; in contrast, *glnN* mRNA was maintained at a level about 45-fold higher than the steady-state level in nitrate-grown cells (Fig. 3C). Experiments with longer incubation times under nitrogen starvation confirmed these results (not shown).

Ammonium does not affect the stability of *glnA* and *glnN* mRNAs. The level of an mRNA reflects the dynamic equilib-

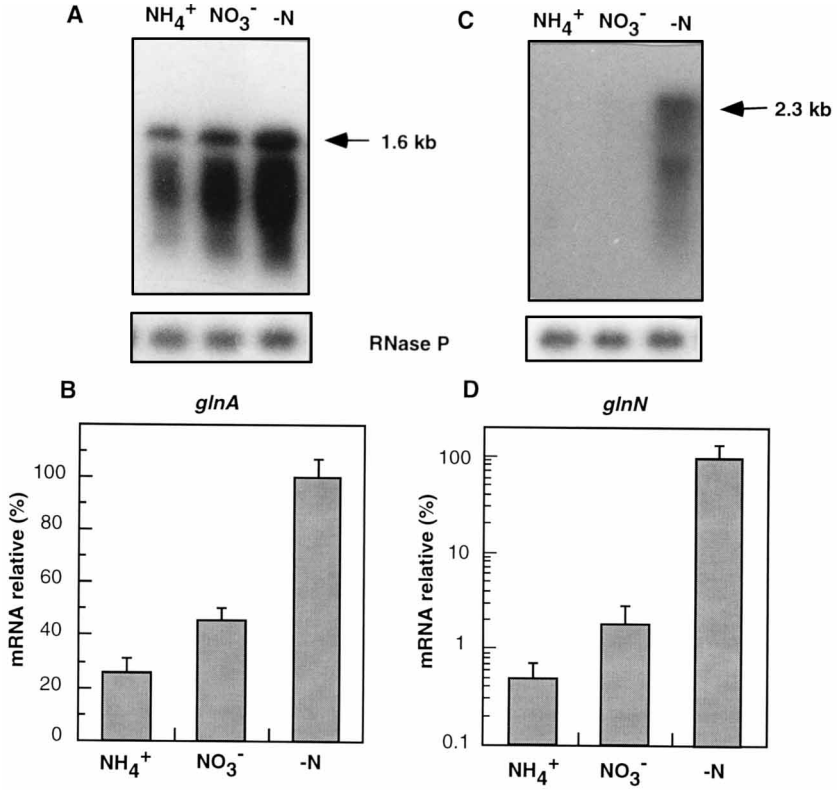


FIG. 2. Levels of *glnA* and *glnN* transcripts in *Synechocystis* sp. strain PCC 6803 cells grown in nitrate- or ammonium-containing medium or under nitrogen deficiency. Total RNA was isolated from mid-log-phase *Synechocystis* sp. strain PCC 6803 cells that utilized nitrate or ammonium as a nitrogen source or from cells subjected to nitrogen deficiency for 12 h. RNA was denatured, electrophoresed in a 1% agarose gel, blotted, and hybridized with a 631-bp *Eco*RV-*Eco*RV internal *glnA* probe (A) or with a 660-bp *Eco*RI-*Eco*RI internal *glnN* probe (C). Autoradiograms of two independent hybridizations were scanned with a densitometer and quantified. (B and D) Average relative values of *glnA* and *glnN* mRNA, respectively. Fifteen micrograms of total RNA was loaded per lane. The filters were stripped and rehybridized with a RNase P RNA gene probe. Transcript sizes were estimated by comparison with 23S, 16S, and 5S rRNAs (34).

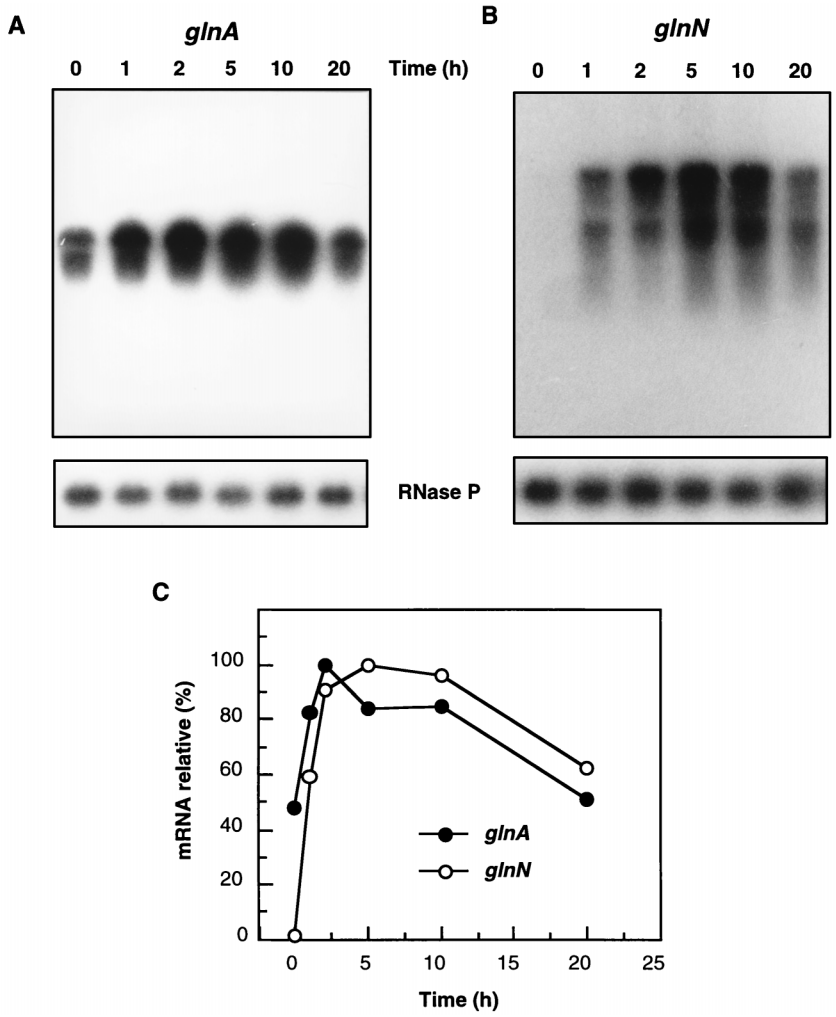


FIG. 3. Kinetics of *glnA* and *glnN* transcript accumulation under nitrogen starvation. Nitrate-grown *Synechocystis* sp. strain PCC 6803 cells were harvested, washed, and transferred to nitrogen-free medium. Samples for total RNA isolation were taken at the indicated times. RNA was processed and hybridized as for Fig. 2. (A) Hybridization using a *glnA* probe. (B) Hybridization using a *glnN* probe. (C) The mRNA levels were quantified by densitometry, and plots of relative mRNA levels versus time were drawn. Values are averages from two hybridization experiments and are expressed as a percentage of the higher value for each transcript.

rium between the rate of synthesis and the rate of decay. In order to investigate whether the observed differences in the amount of *glnA* and *glnN* mRNAs were due to transcriptional regulation of the genes or to differential stability of the transcripts, we determined the half-lives of both transcripts under nitrogen starvation (maximal accumulation condition) or after ammonium addition. For that purpose, nitrate-grown *Synechocystis* sp. strain PCC 6803 cells were transferred to nitrogen-free medium for 10 h, then rifampin (400 μg/ml) or ammonium (1 mM) was added to the culture, and samples for RNA isolation were taken at time intervals thereafter. The decay of both transcripts was monitored by Northern hybridization, and plots of relative mRNA levels versus time were drawn (Fig. 4). For the *glnA* transcript the calculated half-life was 2.5 min after rifampin addition and 3.4 min after ammonium addition (Fig. 4A). In the latter case the final transcript level, 10 min after ammonium addition, was 25%, which is the steady-state level in ammonium-containing medium (Fig. 4C). For the *glnN* mRNA the calculated half-life was 1.9 min after rifampin addition and 1.4 min after ammonium addition (Fig. 4B and D). From these results it can be concluded that differences in the amount of mRNA for both genes under different nitrogen conditions are not caused by differences in the stability of the transcripts, suggesting that *glnA* and *glnN* are regulated mainly at the transcriptional level.

Functional analysis of the *glnA* and *glnN* regulatory regions. In order to determine the minimal DNA fragment containing the promoter activity and the regulatory sequences of *glnA* and *glnN* genes, we have subcloned different portions of the upstream regions of both genes into pFF11. This is a promoter-probe plasmid based on the *cat* (CAT-encoding) reporter gene and constructed for testing of promoters in *Synechocystis* sp. strain PCC 6803 (14). pFF11-derived plasmids that contained fragments from nucleotides −50 to −716 (pJCRP1), to −365 (pJCRP2), or −207 (pJCRP3) or from −199 to −716 (pJCRP4) with respect to the first translated nucleotide of the *glnA* gene were used to transform the SFCΩ5 strain of *Synechocystis* sp. strain PCC 6803, generating strains SJCR11 to SJCR14, respectively (Fig. 5; Table 1). Strains SJCR11, SJCR12, and SJCR13 showed high levels of CAT activity in ammonium-containing medium and twofold higher levels in nitrate-containing medium. When nitrate-grown cells were transferred to nitrogen-free medium, the level of CAT activity increased about threefold after 12 h (Fig. 5). Strain SJCR14

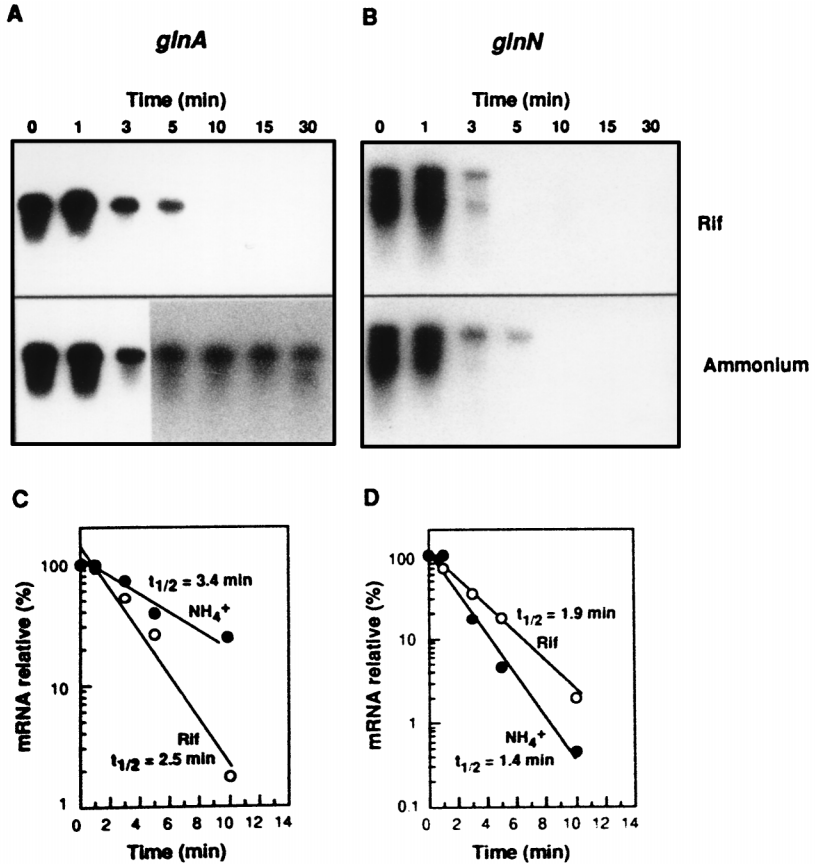


FIG. 4. Effect of ammonium on the stability of *glnA* and *glnN* mRNAs. Nitrate-grown *Synechocystis* sp. strain PCC 6803 cells were transferred to nitrogen-free medium for 10 h. Then rifampin (Rif) (400 μg/ml) or ammonium (1 mM) was added to the culture. Samples for RNA isolation were taken at several times. The decay of the *glnA* (A) and *glnN* (B) transcripts was monitored by Northern blotting. The mRNA levels were quantified by densitometry of two independent hybridizations. Linear regression analysis was performed, and plots of relative mRNA levels versus time were drawn. The half-lives ($t_{1/2}$) of the *glnA* and *glnN* transcripts were calculated from these plots (C and D).

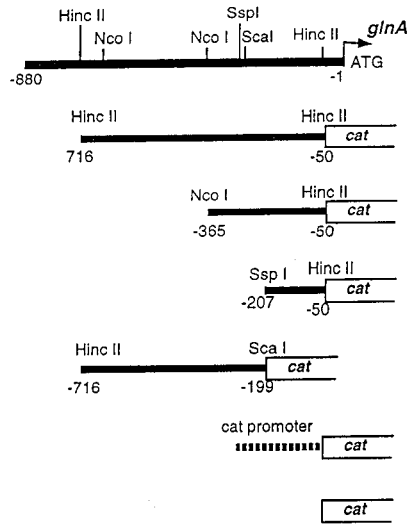
showed low and not regulated CAT activity under all the conditions tested. These results suggested that the *Ssp*I-*Hin*cII fragment (−207 to −50 with respect to the first translated nucleotide) of the *glnA* gene, cloned in the pJCRP3 plasmid, contained the main promoter of the *glnA* gene and most probably the regulatory sequences responsible for the transcriptional regulation. The CAT activity levels of strains SFC57 (Cm^r) and SFF16 (Cm^s) were not affected by the nitrogen conditions (Fig. 5).

We have previously reported the construction of pJCR15(+) and pJCR15(−) plasmids that contain an about 700-bp fragment upstream of the *glnN* gene (41). We have now used pJCR17, a derivative plasmid from pJCR15(+) (see Materials and Methods for details), to generate deletions of the 5′ upstream region of the *glnN* gene. The resulting plasmids contained fragments from nucleotides +191 to −311 (pJCR17.1), to −111 (pJCR17.2), or to +20 (pJCR17.3) with respect to the first translated nucleotide. These plasmids were used to transform the SFCΩ5 strain, generating strains SJCR17.1, SJCR17.2, and SJCR17.3 (Fig. 6; Table 1). When cells of strains SJCR15(+), SJCR17.1, and SJCR17.2 were grown in ammonium- or nitrate-containing medium, no or very low CAT activity was observed; however, CAT activity increased about 100-fold (with respect to the level in nitrate-grown cells) after 12 h of nitrogen deprivation. No CAT activity was detected in SJCR15(−) and SJCR17.3 cells. These results indicated that the promoter and the regulatory sequences of *glnN* gene are contained in the fragment from −111 to +191 inserted in pJCR17.2.

The low CAT activity of strain SJCR15(+) previously reported (41) may be attributed to a mixed population between resident and recombinant plasmids. In fact, after transformation of *Synechocystis* strain SFCΩ5 by an incoming plasmid, the transformants harbor two different plasmids: the recipient one and the recombinant between the incoming and the recipient plasmids (7). To avoid this problem, transformant cells were cured of the endogenous plasmid (pFCΩ5) (see Materials and Methods).

In order to compare the induction properties of both the *glnA* and *glnN* promoter regions, we have studied the kinetics of CAT activity induction after nitrogen deprivation in strains SJCR11 and SJCR15(+). As shown in Fig. 7, the *glnN* promoter [strain SJCR15(+)] responded faster and more strongly than the *glnA* promoter (strain SJCR11) to the nitrogen deficiency. Indeed, after 2 h of nitrogen deprivation CAT activity increased about 10-fold in the SJCR15(+) strain (*glnN* promoter) but only 1.2-fold in the SJCR11 strain (*glnA* promoter). This difference in kinetics of induction suggested differences in the structure of both promoters.

Transcriptional start site mapping. Primer extension analysis was carried out to determine the transcription start point (TSP) of both the *glnA* and *glnN* genes. When an oligonucle-

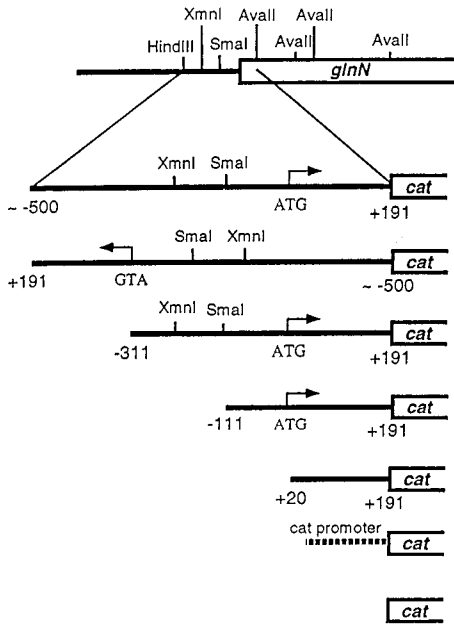


Plasmid	CAT activity in *E. coli*	CAT activity of *Synechocystis* strains with the following nitrogen sources			
		Strain	Nitrate	Ammonium	None
pJCRP1	40 ± 5	SJCR11	615 ± 40	388 ± 30	1840 ± 90
pJCRP2	35 ± 4	SJCR12	607 ± 27	380 ± 35	1700 ± 108
pJCRP3	38 ± 5	SJCR13	637 ± 42	350 ± 39	1850 ± 119
pJCRP4	85 ± 9	SJCR14	75 ± 15	80 ± 12	100 ± 19
pFC57	2270 ± 179	SFC57	1023 ± 123	1000 ± 95	1200 ± 150
pFF11	n.d.	SFF16	n.d.	n.d.	n.d.

FIG. 5. Functional analysis of the *glnA* upstream region. Several fragments of the *glnA* 5′ upstream region were cloned in the pFF11 plasmid in front of a promoterless *cat* gene. The distance from the translation initiation site is indicated. Levels of CAT activity in *E. coli* DH5α transformed cells or in *Synechocystis* sp. strain PCC 6803 cells were determined as described in Materials and Methods. *E. coli* cells were grown overnight in Luria-Bertani medium supplemented with 50 μg of kanamycin/ml. *Synechocystis* sp. strain PCC 6803 cells were grown in ammonium- or nitrate-containing medium or transferred for 12 h to nitrogen-free medium. Strain SFF16, which contains a promoterless *cat* gene, and strain SFC57, which contains the *cat* gene under the control of its own promoter, were used as controls. Means and standard errors from two independent experiments are shown. n.d., not detected. The nucleotide numbers are with respect to the translation start site.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Source or reference
Synechocystis sp. strain PCC 6803		
WT		Pasteur Culture Collection
SFCΩ5	Sr^r; WT transformed with pFCΩ5	7
SFC57	Sr^r Km^r Cm^r; SFCΩ5 transformed with pFC57	7
SFF16	Sr^r Km^r; SFCΩ5 transformed with pFF11	14
SJCR3	Km^r *glnA*::*npt*	41
SJCR6	Km^r *glnN*::*npt*	41
SJCR11	Sr^r Km^r; SFCΩ5 transformed with pJCRP1	This work
SJCR12	Sr^r Km^r; SFCΩ5 transformed with pJCRP2	This work
SJCR13	Sr^r Km^r; SFCΩ5 transformed with pJCRP3	This work
SJCR14	Sr^r Km^r; SFCΩ5 transformed with pJCRP4	This work
SJCR15(+)	Sr^r Km^r; SFCΩ5 transformed with pJCRP15+	39
SJCR15(−)	Sr^r Km^r; SFCΩ5 transformed with pJCRP15−	39
SJCR17.1	Sr^r Km^r; SFCΩ5 transformed with pJCRP17.1	This work
SJCR17.2	Sr^r Km^r; SFCΩ5 transformed with pJCRP17.2	This work
SJCR17.3	Sr^r Km^r; SFCΩ5 transformed with pJCRP17.3	This work
E. coli		
DH5α	F$^-$ *endA1 hsdR17*(m_K^+ r_K^-) *supE44 thi-1 recA1 gyrA96 relA1* (f8d-*lacZ*ΔM15)	20
MC1061	*hsdR mcrB araD139* Δ(*araABC-leu*)*7679* Δ*lacX74 galU galK rpsL thi*	29
Plasmids		
pJCR3	*glnA* in pBluescript II SK(+)	41
pBE2	*glnN* in pBluescript II SK(+)	41
pFF11	Promoter-probe plasmid	14
pJCRP1	*Hin*cII fragment of pJCR3 (666 bp) in pFF11	This work
pJCRP2	*Nco*I-*Hin*cII (315-bp) fragment of pJCR3 in pFF11	This work
pJCRP3	*Ssp*I-*Hin*cII (143-bp) fragment of pJCR3 in pFF11	This work
pJCRP4	*Hin*cII-*Sca*I (517-bp) fragment of pJCR3 in pFF11	This work
pJCR15(+)	*Ava*II-*Hin*dIII fragment of pBE2 in pFF11	41
pJCR15(−)	As pJCR15(+) but with inverted insert	41
pJCR17	Insertion of pBluescript polylinker in pJCR15(+)	This work
pJCR17.1	*glnN* upstream sequence up to −311 in pFF11	This work
pJCR17.2	*glnN* upstream sequence up to −111 in pFF11	This work
pJCR17.3	*glnN* upstream sequence up to +20 in pFF11	This work



PLASMID	CAT activities in *E. coli*	CAT activities of *Synechocystis* strains with the following nitrogen source			
		STRAIN	Nitrate	Ammonium	None
pJCR15(+)	20 ± 5	SJCR15(+)	9 ± 1	n.d.	810 ± 90
pJCR15(-)	n.d.	SJCR15(-)	n.d.	n.d.	n.d.
pJCR17.1	22 ± 4	SJCR17.1	8 ± 2	n.d.	760 ± 87
pJCR17.2	21 ± 3	SJCR17.2	7 ± 2	n.d.	681 ± 77
pJCR17.3	n.d.	SJCR17.3	n.d.	n.d.	n.d.
pFC57	2270 ± 190	SFC57	1123 ± 103	1040 ± 98	1278 ± 150
pFF11	n.d.	SFF16	n.d.	n.d.	n.d.

FIG. 6. Functional analysis of the *glnN* upstream region. Conditions of the experiments are the same than those described in the legend to Fig. 5. The nucleotide numbers are with respect to the translation start site.

otide complementary to the *glnA* mRNA was used as a primer, two contiguous extension products were detected with RNA from cells incubated in nitrate-, ammonium-, or nitrogen-free media (Fig. 8A). The start of transcription mapped to nucleotides −47 and −48 with respect to the first translated nucleotide. −10 (TACGAT) and −35 (TTTTCT) sequences with four and three, respectively, of six sites matching the −10 (TATAAT) and −35 (TTGACA) boxes of the *E. coli* σ^{70}-consensus-like promoters (21) were found upstream of the *glnA* TSP (Fig. 8B). A $GTAN_8TAC$ sequence matching the consensus for the previously defined NtcA-binding site was detected 22 nucleotides upstream of the −10 box (Fig. 8B). These data agree with the results obtained by transcriptional fusions, since the promoter and the putative regulatory sequences are included in the 158-bp *Ssp*I-*Hin*cII fragment of pJCRP3 (Fig. 5 and 8B). Two sequences (TAGAAT and TTG GCA) with only a single mismatch with respect to the −10 and −35 boxes of the σ^{70}-dependent *E. coli*-like promoters and sequence spacing of 16 nucleotides are found 260 bp upstream from the translation start codon of the *glnA* gene. However, no extension product was detected around this region in primer extension experiments. This putative promoter may be responsible for the CAT activity observed in the SJCR14 strain (Fig. 5).

When an oligonucleotide complementary to the *glnN* mRNA was used as a primer, only one extension product was detected; it was much more abundant with RNA from nitrogen-starved cells and only weakly detectable or undetectable with RNA from nitrate- or ammonium-grown cells, respectively (Fig. 9A). The TSP was located at nucleotide −32 with respect to the first translated nucleotide. Sequences with homology to the −10 and −35 boxes of the *E. coli* σ^{70} consensus

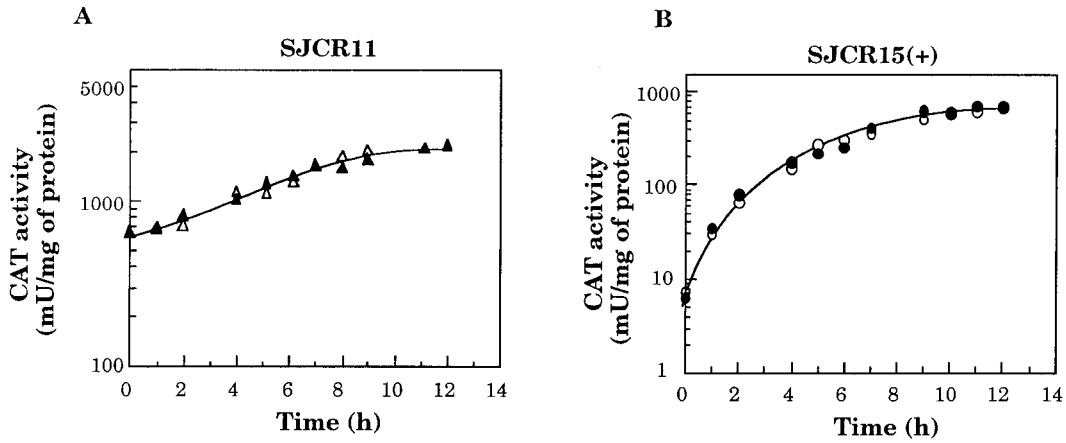


FIG. 7. Kinetics of CAT induction from SJCR11 (*glnA-cat* fusion) and SJCR15(+) (*glnN-cat* fusion). Nitrate-grown SJCR11 and SJCR15(+) cells were washed and transferred at time zero to nitrogen-free medium. Samples were taken at the indicated times for CAT activity determination. Values from two independent experiments are represented.

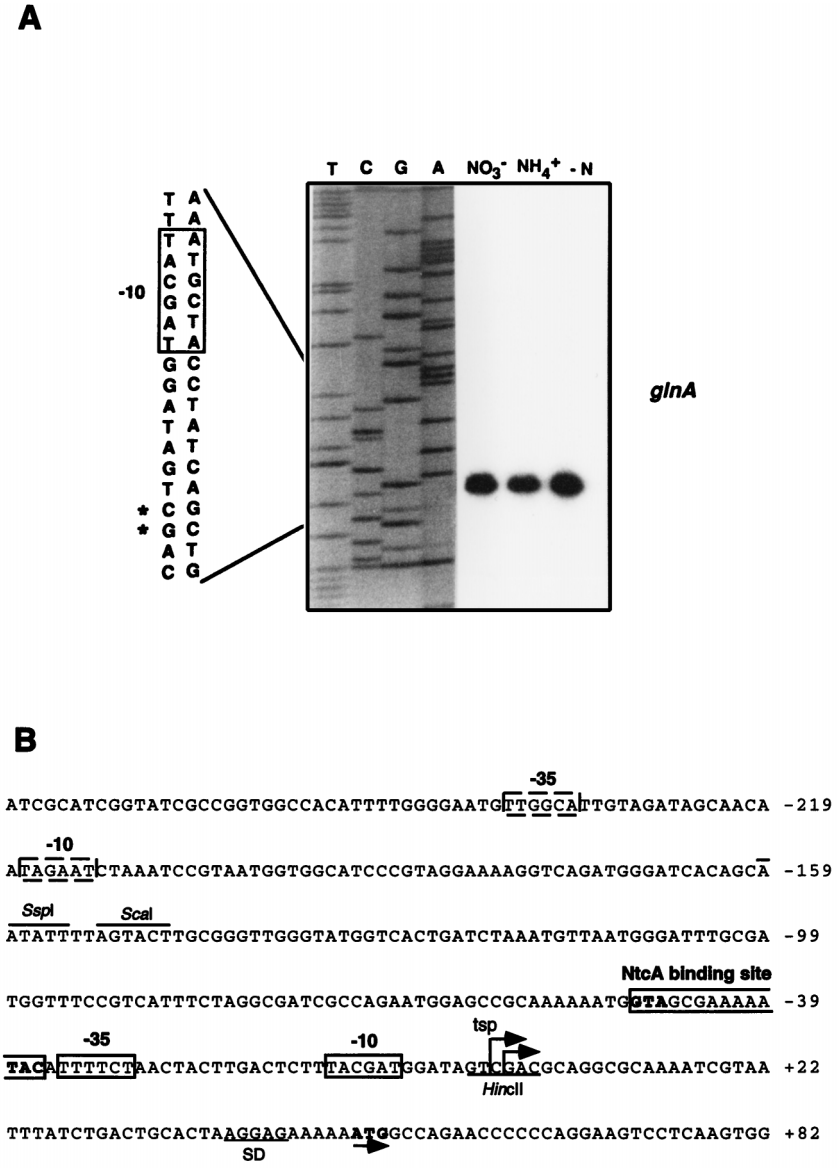


FIG. 8. (A) Primer extension analysis of the *glnA* transcript. Total RNA (50 μg) from ammonium- or nitrate-grown cells or from nitrogen-starved cells was annealed to an oligonucleotide of the *glnA* gene and extended with avian myeloblastosis virus reverse transcriptase as described in Materials and Methods. Lanes T, C, G, and A contain a dideoxy sequencing ladder carried out with the same primer. Transcription start nucleotides are indicated by asterisks. (B) Promoter region of the *glnA* gene. The start codon of the translation is indicated in boldface type. A putative Shine-Dalgarno (SD) sequence is underlined. The TSP is indicated by arrows. −10 and −35 sequences based on the transcriptional start site are boxed with continuous lines. −10 and −35 sequences based on similarity with *E. coli* consensus sequences are boxed with dotted lines. The putative NtcA binding site is also indicated. Also shown are the restriction sites used for *cat* transcriptional fusions. The nucleotide numbers are with respect to the transcription start site.

promoters were located upstream of the TSP (TATGAT and TTGTCT) (Fig. 9B). However, the distance separating the −35 and the −10 sites is 21 bp, distant from the optimal 17 bp. Interestingly, 22 nucleotides upstream of the −10 box, a sequence related to the NtcA binding site ($GTAN_8GTC$ instead of $GTAN_8TAC$) was found (Fig. 9B).

Binding of NtcA to the promoter of the *Synechocystis* sp. strain PCC 6803 *glnA* gene. In order to test whether the transcription activator NtcA binds to the promoter of both the *glnA* and *glnN* genes, we have performed mobility shift experiments with purified *Synechocystis* NtcA protein. *Synechocystis* NtcA was expressed in *E. coli* and purified as a GST fusion protein as previously described (36). As shown in Fig. 10A, purified NtcA retarded an *Ssp*I-*Hinc*II 158-bp fragment that contains the *glnA* promoter (from −157 to +1 with respect to the TSP [Fig. 8]). NtcA-promoted shift was severely diminished in the presence of an excess of the same unlabeled fragment, and it was unaffected by the presence of an excess of an unrelated DNA fragment (not shown). The GST protein expressed with the same vector and purified by the same protocol was unable to retard the fragment containing the *glnA* gene promoter. The equilibrium dissociation constant (K_d) of the binding reaction determined from NtcA saturation experiments (Fig. 10B) was 25×10^{-9} M at 25°C. These data suggest that the NtcA protein specifically binds to the *glnA* promoter of *Synechocystis* sp. strain PCC 6803.

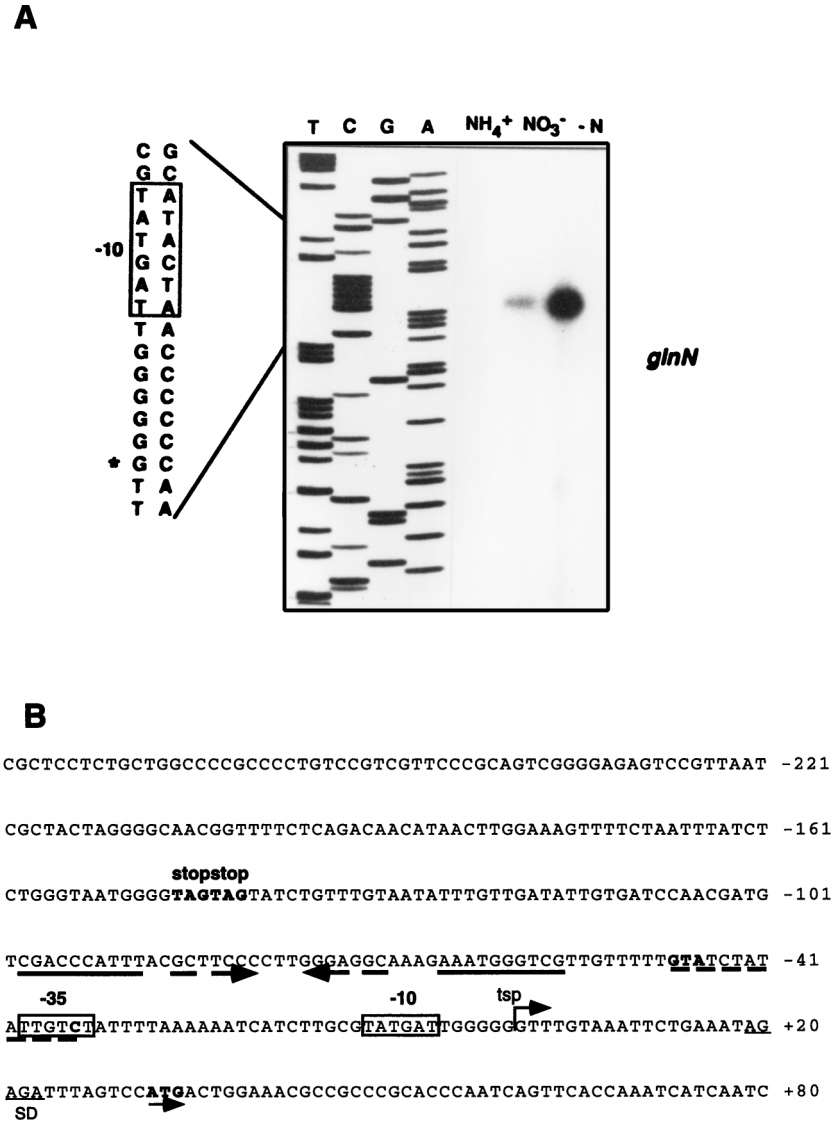


FIG. 9. (A) Primer extension analysis of the *glnN* transcript. Total RNA (50 μg) from ammonium- or nitrate-grown cells or from nitrogen-starved cells was annealed to an oligonucleotide of the *glnN* gene and extended with avian myeloblastosis virus reverse transcriptase as described in Materials and Methods. Lanes T, C, G, and A contain a dideoxy sequencing ladder carried out with the same primer. Transcription start nucleotides are indicated by asterisks. (B) Promoter region of the *glnN* gene. The start codon of the translation is indicated in boldface type. A putative Shine-Dalgarno (SD) sequence is underlined. The TSP is indicated by an arrow. −10 and −35 sequences based on the transcriptional start site are boxed. A sequence similar to the consensus NtcA binding sequence is underlined with a dotted line. A palindromic inverted repeat motif is underlined with boldfaced arrows. The nucleotide numbers are with respect to the transcription start site.

As mentioned above, we found a putative degenerate NtcA binding site upstream of the −10 box of the *glnN* promoter (Fig. 9). Therefore, the binding of NtcA to different fragments containing the promoter of the *glnN* gene was investigated. NtcA did not bind to these fragments in the mobility shift assay (data not shown).

DISCUSSION

We have previously reported that in nitrate-grown *Synechocystis* sp. strain PCC 6803 cells, the *glnA* gene product (GSI) is responsible for 97% of the total GS activity, while the *glnN* product (GSIII) accounts for only about 3%. However, after 24 h of nitrogen deprivation, the activity corresponding to the GSIII represents about 20% of the total GS activity (41) (Fig. 1). Northern blot and transcriptional gene fusion experiments reported in this work indicate that expression of *glnA* and *glnN* genes is differentially regulated in response to changes in nitrogen availability. Indeed, the *glnA* gene presents a high basal level of expression in ammonium-grown cells that increases about two- to fourfold in nitrate-grown or nitrogen-starved cells, respectively (Fig. 2A and B and 5). However, *glnN* gene expression only was strongly up-regulated in nitrogen-starved cells, reaching levels close to 100-fold higher than those determined in nitrate- or ammonium-growing cells (Fig. 2C and D and 6). In addition, Northern experiments also showed that after long nitrogen starvation periods (20 h or more), the level of *glnA* transcript returned to the steady-state level found in nitrate-grown cells while the level of *glnN* mRNA remained high (Fig. 3). All these data strongly suggest that while *glnA* gene encodes the housekeeping GS and its expression is necessary under all growing conditions, *glnN* expression is specif-

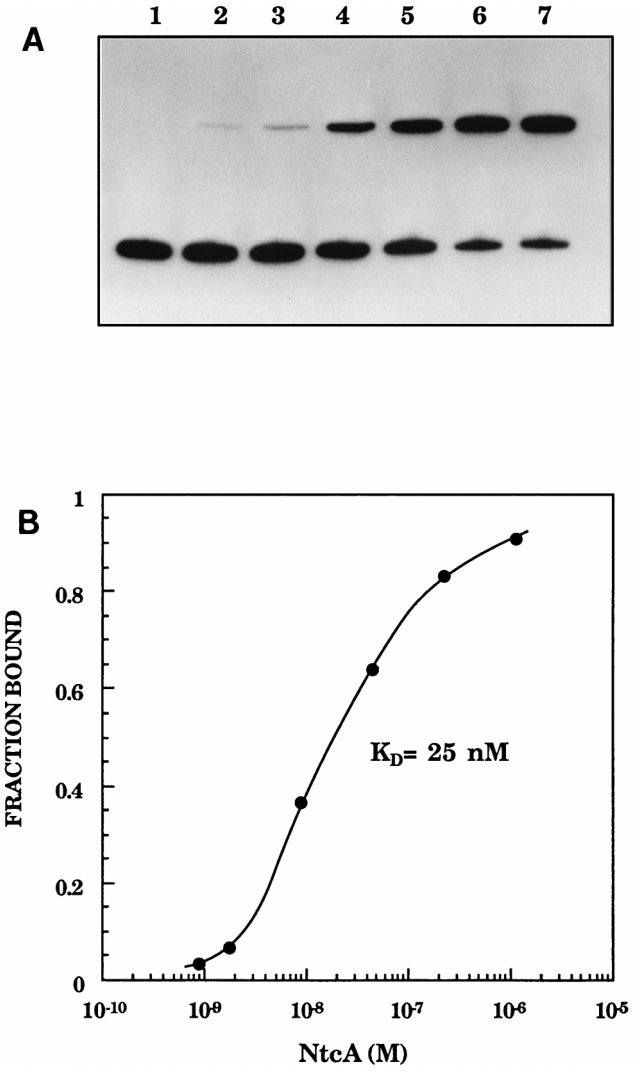


FIG. 10. Binding of NtcA to the *Synechocystis* sp. strain PCC 6803 *glnA* gene promoter. (A) A 158-bp DNA fragment encompassing the *glnA* promoter was incubated in the presence of various concentrations of purified NtcA protein. Lane 1, no protein; lane 2, 0.9 nM NtcA; lane 3, 1.8 nM NtcA; lane 4, 9 nM NtcA; lane 5, 45 nM NtcA; lane 6, 0.225 μM NtcA; lane 7, 1.125 μM NtcA. (B) Bound and free DNAs were quantified in a Molecular Dynamics PhosphorImager, and the fraction of bound probe was plotted versus NtcA concentration.

ically required under nitrogen deficiency. It is worth noting that deprivation of other essential macronutrients such as sulfur or phosphorus did not increase the expression of the *glnA* or *glnN* gene (data not shown).

The family *Rhizobiaceae* contain at least two different forms of GS: the classical prokaryotic GSI, encoded by the *glnA* gene, and GSII, similar to the eukaryotic GS, encoded by the *glnII* gene (5). In most of the *Rhizobiaceae* so far studied, the *glnA* gene is constitutively expressed (10), while the transcription of *glnII* is activated by an Ntr-like system under nitrogen-limiting conditions (6, 12, 45). Therefore, a clear parallelism exists between *Synechocystis* sp. strain PCC 6803 and *Rhizobiaceae* with respect to the regulation of GS genes. In both cases, expression of GSI is not strongly regulated while expression of the alternative GS (GSII in the case of *Rhizobiaceae* and GSIII in the case of *Synechocystis* strain PCC 6803) is subjected to a strict control in response to nitrogen source availability.

Our results also indicate that expression of the *glnA* and *glnN* genes is mainly controlled at the level of transcription initiation, since half-lives of both transcripts were almost the same under nitrogen starvation or after addition of ammonium to nitrogen-starved cells (Fig. 4). In prokaryotic cells mRNA stability may vary over a wide range, contributing to the differential expression of individual genes (37). The half-lives of *E. coli* mRNAs vary from less than 0.5 to more than 20 min, with an average half-life of 2 to 4 min. However, in cyanobacteria the average half-life of total mRNA is approximately 12 to 20 min (23, 57), and the few values for specific transcripts so far described fall into this range (2, 35). This long half-life of cyanobacterial mRNAs has been related to the long doubling time of cyanobacteria compared to enterobacteria (about 8 h versus about 30 min). Therefore, half-lives of *glnA* and *glnN* messengers of *Synechocystis* are exceptionally short (2.5 min for *glnA* and 1.9 min for *glnN*). This short mRNA half-life implies the capacity for a rapid response to environmental change, suggesting that *Synechocystis* has evolved mechanisms to respond quickly to the presence of ammonium. It is worth noting that *Synechocystis* GSI is rapidly inactivated after addition of ammonium to the medium, the enzyme being reactivated when the ammonium is consumed (31, 32, 42).

A discrepancy exists between the level of induction of the GSIII activity (about 10-fold) (Fig. 1) (41) and the induction of *glnN* transcription (90- to 100-fold) (Fig. 2, 3, and 6), suggesting that a posttranscriptional regulation is involved in the modulation of GSIII expression. Western blot experiments also revealed an approximately 10-fold induction in the quantity of GSIII protein (data not shown). Since we have demonstrated that stability of the *glnN* mRNA is similar in the presence or absence of a nitrogen source, control of the amount of GSIII protein should be also regulated at the level of mRNA translation or protein stability.

Functional analysis of the *glnA* promoter region showed that regulated expression of CAT activity required DNA sequences between −207 and −50 with respect to the translation start point (Fig. 5). Primer extension experiments confirmed that the *glnA* promoter is located in this fragment. The structure of *glnA* promoter corresponds precisely to the structure of the NtcA-regulated promoters as defined for *Synechococcus* sp. strain PCC 7942 genes by Luque et al. (24) (Fig. 8). Indeed, purified *Synechocystis* NtcA protein binds specifically to DNA fragments containing this region. These data suggest that the *glnA* gene of *Synechocystis* is positively regulated by the binding of the NtcA protein to its promoter. On the other hand, NtcA-regulated promoters do not require a −35 box (24). However, in the *Synechocystis glnA* promoter we found a sequence (TT TTCT) which adapts to the −35 box of the *E. coli* σ^{70} consensus promoters. This is most probably responsible for the high basal level of expression of this promoter in the presence of ammonium, when NtcA is not active.

Differences between the *glnA* and *glnN* promoters in the basal transcription level and in the kinetics and level of induction suggested the existence of differences in their structure and regulatory sequences. Analysis of the *glnN* promoter indicated that the low basal level of expression could be attributed to the long spacing between the −10 and −35 boxes (21 bp instead of the optimal 17 bp) (Fig. 9). The fact that a sequence weakly related to the consensus NtcA binding site exists at an appropriate position upstream of the *glnN* gene suggested that this factor is involved in the regulation of *glnN* transcription. However, *E. coli*-expressed NtcA protein does not bind, in vitro, to this region. Three alternative possibilities could explain our results. First, NtcA does not bind in vivo to the *glnN* promoter, and other factors are involved in the transcriptional

regulation of this gene. Second, NtcA binds to the *glnN* promoter with much less affinity than to the *glnA* promoter, and we are not able to detect the binding. Therefore, activation of the *glnN* promoter would require a higher concentration of NtcA protein than activation of the *glnA* promoter. Since *ntcA* gene transcription is autoregulated (24), the level of NtcA increases progressively during nitrogen starvation. If the previous hypothesis is true, we should expect for the *glnA* promoter a faster induction than for the *glnN* promoter, after the shift to nitrogen-free medium. However, the opposite result is observed in the kinetics of induction of CAT activities shown in Fig. 7, suggesting that this possibility is unlikely. The third possibility is that binding of NtcA to the *glnN* promoter requires the presence of another factor that is not present in the in vitro band shift assay. This putative factor would be required only for the binding of NtcA to a subset of nitrogen-regulated promoters.

In conclusion, our results clearly demonstrate that both *Synechocystis* GS-encoding genes are transcriptionally regulated, depending on the nitrogen availability to the cells. However, the regulation patterns exhibited by both genes are different and suggest that *glnA* encodes the housekeeping GS while *glnN* encodes a nitrogen starvation-specific GS. In nature, cyanobacteria are probably subjected to long nitrogen starvation periods. The *glnN* expression would be induced under those conditions, and GSIII would be involved to a significant extent in the assimilation of the little nitrogen available. Why the *glnN* gene product, GSIII, is specifically required under nitrogen deficiency and the nature of the transcription factor that regulates its expression are questions that will require further studies.

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