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The NADP$^+$-Isocitrate Dehydrogenase Gene (*icd*) Is Nitrogen Regulated in Cyanobacteria

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NADP$^+$-isocitrate dehydrogenase (NADP$^+$-IDH) activity and protein levels in crude extracts from the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 and the filamentous, dinitrogen-fixing *Anabaena* sp. strain PCC 7120 were determined under different nitrogen conditions. The highest NADP$^+$-IDH activity and protein accumulation were found under dinitrogen-fixing conditions for the *Anabaena* strain and under nitrogen starvation for *Synechocystis* sp. PCC 6803. The *icd* gene that encodes the NADP$^+$-IDH from *Synechocystis* sp. strain PCC 6803 was cloned by heterologous hybridization with the previously isolated *icd* gene from *Anabaena* sp. strain PCC 7120. The two cyanobacterial *icd* genes show 81% sequence identity and share a typical 44-amino-acid region different from all the other *icd* genes sequenced so far. The *icd* gene seems to be essential for *Synechocystis* growth since attempts to generate a completely segregated *icd* mutant were unsuccessful. Transcripts of 2.0 and 1.6 kb were detected by Northern (RNA) blot analysis, for the *Anabaena* and *Synechocystis icd* genes, respectively. Maximal *icd* mRNA accumulation was reached after 5 h of nitrogen starvation in *Synechocystis* cells and under dinitrogen-fixing conditions in *Anabaena* cells. Primer extension analysis showed that the structure of the *Synechocystis icd* gene promoter resembles those of the NtcA-regulated promoters. In addition, mobility shift assays demonstrated that purified *Synechocystis* NtcA protein binds to the promoter of the *icd* gene. All these data suggest that the expression of the *icd* gene from *Synechocystis* sp. strain PCC 6803 may be subjected to nitrogen control mediated by the positively acting regulatory protein NtcA.

Cyanobacteria are photosynthetic prokaryotes that have an incomplete tricarboxylic acid cycle, lacking α-ketoglutarate dehydrogenase and succinyl-coenzyme A synthetase activities (46, 54). The NADP$^+$-isocitrate dehydrogenase (IDH) (EC 1.1.1.42) reaction represents a terminal step in carbon flow; thus, the role of this enzyme is the provision of biosynthetic precursors rather than energy production. The α-ketoglutarate produced in the NADP$^+$-IDH reaction is required for ammonium assimilation through the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (38) and is a key metabolite in the linking of nitrogen and carbon metabolism.

IDHs have been purified and characterized from a variety of sources (8); most bacteria have only an NADP$^+$-dependent IDH consisting of two identical subunits with molecular weights of between 40,000 and 57,000 (8). There are other prokaryotic NADP$^+$-IDHs that are monomeric enzymes with molecular weights of about 80,000 (13, 33). In some cases, both NADP$^+$-IDH types coexist in the same organism, as in *Vibrio* sp. strain ABE-1 (24).

The genes coding for different IDHs have been cloned and sequenced from prokaryotic and eukaryotic sources (10, 11, 13, 19, 20, 22, 25, 26, 37, 41, 45, 53). Comparison of the deduced amino acid sequences revealed conserved regions among dimeric and among monomeric IDHs (45), but no similarity could be detected between both groups of IDHs (13).

In cyanobacteria, IDH is strictly dependent on NADP$^+$ and belongs to the typical dimeric type. The enzyme has been purified and characterized from the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (44) and the filamentous dinitrogen-fixing *Anabaena* sp. strain PCC 7120 (45). In both cases, the enzymes show kinetic and physicochemical parameters similar to those of the NADP$^+$-IDH from *Escherichia coli* (44, 45, 48). The *Anabaena icd* gene has been cloned by complementation of an *E. coli icd* mutant (45). The deduced sequence of *Anabaena* NADP$^+$-IDH is similar to those of other prokaryotic NADP$^+$-IDHs but presents an extra region which seems to be specific for the cyanobacteria (45).

In bacteria that have a complete Krebs cycle, like *E. coli*, the α-ketoglutarate produced through the IDH reaction can be further oxidized within the cycle or reductively aminated to glutamate. Thus, this metabolite may be implicated in energy production or in biosynthetic reactions, depending on cellular needs. On the other hand, in organisms able to use acetate as sole carbon source, the IDH has an important role in controlling the carbon flow at the branch point of the glyoxylate bypass and the Krebs cycle. In *E. coli*, NADP$^+$-IDH activity is regulated by phosphorylation of the enzyme when cells are growing with acetate, which partially inactivates the NADP$^+$-IDH and allows the cell to maintain the supply of carbon compounds required for the synthesis of cellular constituents (32).

In cyanobacteria, as the α-ketoglutarate produced in the IDH reaction cannot be further oxidized, it directly enters the GS-GOGAT cycle and has a clearly biosynthetic role related to nitrogen assimilation (46).

Little is known about the transcriptional regulation of prokaryotic *icd* genes. In *Bacillus subtilis*, in which the IDH gene (*citC*) is in a single transcription unit together with one of the citrate synthase genes (*citZ*), the expression of the operon is maximal at the end of the exponential growth phase, being repressed by the presence of glutamate and/or glucose (26, 27).

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In *Vibrio* sp. strain ABE-1, *icdI* and *icdII* genes, coding for a dimeric and a monomeric IDH, respectively, are regulated differently at the transcriptional level. The *icdII* mRNA level is increased by lowering the growth temperature while *icdI* mRNA is affected by the carbon source (25, 55).

In this work, we report that expression of the *icd* gene is subjected to nitrogen control in the cyanobacteria *Synechocystis* sp. strain PCC 6803 and *Anabaena* sp. strain PCC 7120. We have also cloned and sequenced the *Synechocystis icd* gene, and the 5′ region of this gene has been studied. Primer extension and band shift experiments suggest that the *Synechocystis icd* promoter is an NtcA-regulated promoter. The transcriptional activator NtcA has been identified as a regulatory element of the nitrogen control mechanism in cyanobacteria (34, 57). Thus, expression of the *icd* gene and that of nitrogen assimilation are coordinated.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Synechocystis* sp. strain PCC 6803 was grown at 30°C with shaking in BG11 medium (50) supplemented with 12 mM $NaHCO_3$ (BG11C). BG11 contains 18 mM NO_3Na as nitrogen source. For nitrogen starvation conditions, nitrate was not added to the medium (named $BG11_0C$ or $BG11_0$ medium, depending on the addition or not of $NaHCO_3$, respectively). For plate cultures, BG11C liquid medium was supplemented with 1% (wt/vol) agar. Chloramphenicol was added to a final concentration of 20 μg/ml when required. For induction experiments, cultures were bubbled with 1.5% (vol/vol) CO_2 in air; when they reached a chlorophyll concentration of 10 μg/ml, cells were harvested, washed twice with $BG11_0C$, and further incubated in this medium. Chlorophyll was measured in methanolic extracts (35).

Synechocystis sp. strain PCC 6803, harboring the pFCΩ5 Sm^r plasmid (SFCΩ5), was used in transformation with the disrupted *icd* gene (7). *Anabaena* sp. strain PCC 7120 was grown at 30°C with shaking, with $BG11_0$, BG11, or $BG11_0$ plus NH_4^+. For RNA extraction purposes, the cultures were grown in the same medium but bubbled with air. In all the cases when ammonium was used as the nitrogen source, nitrate was replaced by 10 mM NH_4Cl and the medium was buffered with 20 mM *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) buffer, pH 7.0.

E. coli DH5α (Bethesda Research Laboratories), used for all plasmid constructions, and *E. coli* MC1061 (39), used for gene library construction, were grown in Luria broth as described elsewhere (51). The medium was supplemented with ampicillin at 100 μg/ml when required.

Cell extracts, enzyme assay, and protein determination. Cells were harvested by centrifugation at 5,000 × *g* for 10 min, resuspended in 30 mM Tris-HCl (pH 7.5), and broken by crushing them in a mortar containing liquid nitrogen. The lysate was centrifuged at 12,000 × *g* for 15 min, and the resulting supernatant constituted the cell extract.

NADP$^+$-specific IDH activity was measured as previously described (44). Units are expressed as micromoles of NADPH produced per minute. Protein concentrations were determined by the method of Bradford (3), with bovine serum albumin as standard.

DNA manipulation and gene sequence. Total DNA from cyanobacteria was isolated as described by Cai and Wolk (4). Plasmid isolation from *E. coli*, transformation of *E. coli*, restriction, and ligation with T4 ligase were performed by standard procedures (1, 51). DNA fragments were purified from agarose gels with the Gene-Clean Kit (Bio 101, Inc.). For Southern hybridizations, DNA was digested and fragments were electrophoresed in 0.7% agarose gels in a Tris-borate-EDTA buffer system (51). Transfer of DNA to Z-Probe membranes (Bio-Rad Laboratories) was done under vacuum, and Southern blot hybridizations were performed as described previously (1). DNA probes were ^{32}P labeled by the random primer technique with [α-^{32}P]dCTP. For heterologous Southern hybridizations, low-stringency conditions (55°C, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) were used and the filters were washed at room temperature.

Sequencing of both strands of the DNA fragments containing the *icd* gene was carried out by the dideoxy-chain termination method (52), with Sequenase version 2.0 (U.S. Biochemical Corp.). Nested unidirectional deletions of pMS1 and pMS3 (an *Eco*RI subclone of pMS2) were generated with the double-stranded Nested Deletion Kit from Pharmacia LKB. The sequence of the 5′ region upstream of the *icd* gene and the nucleotides coding for the first four amino acids of the IDH protein were determined by extension of the primer: 5′ ATCGTT GGGTACTACGGGCT 3′ (from nucleotides 78 to 59 of the coding region). This primer was also used for the mapping of the transcriptional start site (see below). The junction between the adjacent *icd* fragments of the plasmids pMS1 and pMS3 was sequenced with the plasmid pMS4 and appropriate oligonucleotides.

Computer searches for homologies were done by using the FASTA program, and alignments were produced with the Pileup program with default parameters (12) and improved by manual alignment.

Western blot (immunoblot) analysis. Crude extracts from *Synechocystis* or *Anabaena* cells, grown under different conditions, were subjected to denaturing electrophoresis on 12% (mass/vol) polyacrylamide gels (30). Western blot procedures were carried out as previously described (44). In all cases, the same amount of total protein was loaded.

Insertional mutagenesis of the *Synechocystis icd* gene. A 1.9-kb DNA fragment, containing a chloramphenicol resistance gene from RSF1010 (14), was cloned into the *Apa*I internal site of the *icd* gene (see Fig. 3) in both orientations. The resulting plasmids, derived from pMS3 (pMS3CmA and pMS3CmB) (see Fig. 3), were used to transform *Synechocystis* SFCΩ5 (7) as previously described (6).

RNA isolation and Northern (RNA) blot analysis. Total RNA from *Synechocystis* sp. strain PCC 6803 was isolated by the method of hot phenol as described by Mohamed and Jansson (42) with the modifications described in reference 49. RNA from *Anabaena* sp. strain PCC 7120 was isolated as described in reference 17. Separation of RNA on formaldehyde gels, transfer to nylon membranes (Hybond N-plus; Amersham), prehybridization, and hybridization conditions were according to the instruction manual from Amersham. A 15-μg sample of total RNA was loaded per lane. Relative transcript levels were quantified with a scanning densitometer (Bio Image; Millipore Corporation) from at least two different autoradiographs. In all the cases, the upper band containing the nondegraded transcript was quantified.

Primer extension analysis. The oligonucleotide used for primer extension was 5′ ATCGTTGGGTACTACGGGCT 3′ (from nucleotide 78 to 59 of the coding region). After end labeling with T4 polynucleotide kinase (Boehringer) and [γ-^{32}P]dATP as described elsewhere (51), 50 ng of labeled oligonucleotide (about 10^6 cpm) was annealed to 50 μg of total RNA from *Synechocystis* sp. strain PCC 6803, grown under different nitrogen conditions, in 15 μl of hybridization buffer (10 mM Tris HCl [pH 8.3], 0.15 M KCl, and 1 mM of EDTA). Mixtures were incubated first at 85°C for 5 min and then at 50°C for 3 h. The extension reactions were carried out at 44°C for 1 h as described previously (1), with 10 U of avian myeloblastosis virus reverse transcriptase (Promega). Reaction mixtures were then treated with RNase A (DNase-free; Boehringer) and extracted with phenol. DNA was precipitated with ethanol, resuspended in formamide-loading dye, and then analyzed on a sequencing gel (6% polyacrylamide). To determine the size of the extension product, nucleotide sequencing of an appropriate plasmid was carried out with the same oligonucleotide as a primer.

Cloning of *Synechocystis* sp. strain PCC 6803 *ntcA* gene and purification of GST-NtcA fusion protein. The complete *ntcA* open reading frame was cloned from *Synechocystis* sp. strain PCC 6803 genomic DNA after PCR amplification with the oligonucleotides M1 (5′ ATACTCGAGATGGATCAGTCCCTAACC 3′) (from nucleotide 1 to 18 of the *ntcA* coding region) and M2 (5′ TCACTC GAGGGCACTGGTCATAGAGG 3′) (from nucleotide 694 to 682 of the *ntcA* coding region). The resulting 715-bp DNA fragment was restricted with *Xho*I and cloned into the *Xho*I site of pGEX-4T-1 plasmid in phase with the glutathione S-transferase (GST) gene to create pGEX-NtcA. The complete *ntcA* gene and the reading frame of the fusion protein were checked by DNA sequencing.

GST-NtcA fusion protein and GST were expressed in *E. coli* LC137 from plasmids pGEX-NtcA and pGEX-4T-1, respectively. One liter of culture was grown in Luria broth medium to an optical density at 600 nm of 0.6, induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 2.5 h, harvested by centrifugation, and resuspended in 5 ml of PBS buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 4 μM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol) supplemented with 1% Triton X-100. Cells were broken by sonication on ice, and insoluble debris was pelleted by centrifugation. Extracts were mixed with 1 ml of glutathione agarose beads (Pharmacia) and incubated for 2 h at 4°C with gentle agitation. Then beads were transferred to a column and washed extensively with PBS buffer until no more protein was eluted from the column. GST or GST fusion proteins were eluted with 3 ml of 50 mM Tris HCl (pH 8) containing 10 mM reduced glutathione.

Gel retardation assays. Probe was isolated from agarose gels after digestion of pMS4 plasmid with *Xmn*I and *Spe*I. The 128-bp fragment was end labeled with [α-^{32}P]dCTP with Sequenase version 2.0 enzyme. The binding reaction was carried out in a final volume of 30 μl containing 4 ng of labeled DNA and 2 μg of poly(dI-dC) in 25 mM Tris HCl (pH 8.0)–50 mM KCl–4 mM spermidine–10% glycerol. To this mixture, 0.2 or 0.4 μg of purified NtcA-thrombin-cleaved protein was added. One microgram of GST-NtcA fusion protein was cleaved by incubation in PBS buffer supplemented with 1 U of thrombin and 2.5 mM $CaCl_2$ for 15 min at 30°C. Binding reactions with 4 μg of GST protein, treated with thrombin under the same conditions, were also performed. For competition experiments, a 30-fold excess of unlabeled probe was added to the binding reaction mixture. As an unrelated competitor DNA, a fragment of pBluescript II SK plasmid was used. The mixtures were incubated at 25°C for 15 min and loaded into a nondenaturing 6% polyacrylamide gel. Electrophoresis was carried out at 4°C and 280 V, and then gels were placed over Whatman 3MM paper, dried, and autoradiographed.

Nucleotide sequence accession number. The EMBL-GenBank accession number for the *Synechocystis icd* sequence described here is X83563.

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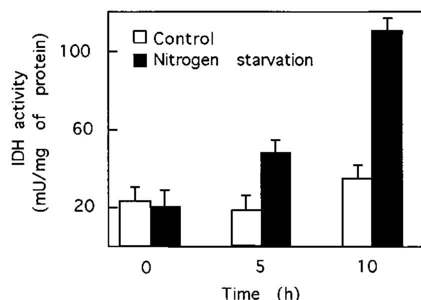

FIG. 1. Effect of nitrogen deficiency on the NADP$^+$-IDH specific activity from *Synechocystis* sp. strain PCC 6803. Nitrate-grown *Synechocystis* cells were washed with nitrogen-free medium and transferred, at time zero, either to nitrogen-free medium (nitrogen starvation) or to nitrate-containing medium (control). Samples were taken at the indicated time, and NADP$^+$-IDH was determined in cell extracts as described in Materials and Methods. Data are the means of three independent experiments, and standard errors are represented by bars.

RESULTS

Effect of nitrogen feeding on IDH activity and protein levels. Since IDH is the enzyme responsible for the α-ketoglutarate supply for ammonium assimilation, through the GS-GOGAT pathway (38), we determined if the levels of IDH activity and the amount of IDH protein were modulated in response to changes in nitrogen availability. Figure 1 shows that the IDH activity of crude extracts from *Synechocystis* sp. strain PCC 6803 cells subjected to nitrogen starvation for 10 h was between three- and fivefold higher than that from nitrate-grown cells. In order to test the amount of IDH protein, crude extracts from different conditions were subjected to Western blot analysis, with polyclonal antibodies against *Synechocystis* sp. strain PCC 6803 IDH (44). The amount of IDH protein increased after 5 or 10 h of nitrate removal from a *Synechocystis* sp. strain PCC 6803 culture (Fig. 2A), indicating that the increase observed in the level of IDH activity corresponded to a higher amount of IDH protein. Similar results were obtained when cells were grown in ammonium-containing medium and then transferred to nitrogen-free medium (not shown). As a control, the activity of another enzyme of carbon metabolism, glucose-6-phosphate dehydrogenase, was tested in crude extracts from *Synechocystis* nitrogen-starved cells. In this case, no significant differences were observed between nitrate-growing cells (55 mU/mg of protein) and nitrogen-deficient cells (60 mU/mg of protein).

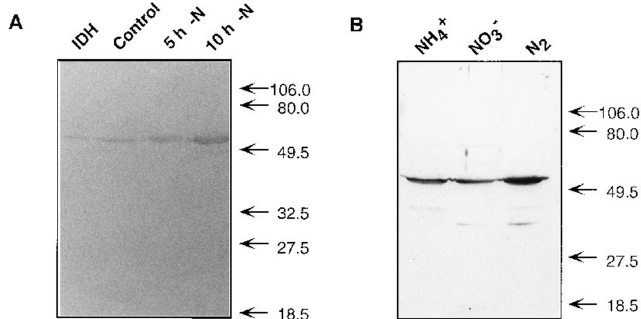

FIG. 2. Effect of nitrogen availability on the amount of NADP$^+$-IDH protein of *Synechocystis* sp. strain PCC 6803 and *Anabaena* sp. strain PCC 7120 cells. (A) Nitrate-grown *Synechocystis* cells were washed and transferred to nitrogen-free medium. At the indicated times, samples were taken for crude extract preparation. A total of 85 μg of protein of each extract or 2 μg of purified NADP$^+$-IDH protein was subjected to Western blot analysis, with polyclonal antibodies against *Synechocystis* IDH. (B) Crude extracts from *Anabaena* sp. strain PCC 7120 cells grown with different nitrogen sources were subjected to Western blot analysis with polyclonal antibodies against *Synechocystis* IDH. A total of 180 μg of protein was loaded per lane. Numbers to the right of each panel show molecular mass in kilodaltons.

In the case of *Anabaena* sp. strain PCC 7120, we had previously reported that the NADP$^+$-IDH activity was higher when the cells were grown under dinitrogen-fixing conditions (44). The activity of crude extracts from ammonium- or nitrate-grown cells represented between 60 and 70% of that from dinitrogen-fixing cells. As shown in Fig. 2B, the higher IDH activity of dinitrogen-fixing cultures corresponded to an increase in the amount of IDH protein. Western blot analysis of *Anabaena* samples was carried out with polyclonal antibodies raised against purified *Synechocystis* NADP$^+$-IDH, reported previously to cross-react with purified *Anabaena* NADP$^+$-IDH (45).

Cloning and sequence of the *Synechocystis icd* gene. To investigate whether the regulation of *Synechocystis* IDH activity, in response to nitrogen starvation, correlated with higher gene expression under these conditions, we isolated the *icd* gene from this cyanobacterium. For this purpose, the previously cloned *icd* gene from *Anabaena* sp. strain PCC 7120 (45) was used as a probe to identify bands hybridizing with digested genomic DNA from *Synechocystis* sp. strain PCC 6803. Several restriction fragments of *Synechocystis* DNA hybridized to the *Anabaena icd* gene. The strategy of constructing a partial genomic library in the pBluescript II SK vector from size-fractionated DNA fragments around a hybridizing band was used, and by this approach, two *Xmn*I-*Xmn*I fragments of 2.4 and 0.5 kb were cloned by colony hybridization. These fragments correspond to the inserts of pMS1 and pMS2, respectively (Fig. 3). However, when we tried to clone the complete *icd* gene from *Synechocystis* sp. by a DNA digestion that gave rise to a single, high-molecular-weight band hybridizing with the *Anabaena* probe, all the attempts were unsuccessful. These results suggested that the cloning in *E. coli* of the *Synechocystis icd* region into a high-copy-number plasmid like pBluescript might be impossible. In fact, the cloning of the *Hinc*II-*Hinc*II, 3.4-kb fragment containing the complete *icd* gene from *Synechocystis* sp. was straightforward, with the low-copy-number pRL500 plasmid (14) as a vector and the same cloning strategy described above (pMS4; Fig. 3).

After sequencing of the appropriate plasmids (see Materials and Methods), one open reading frame of 1,425 bp was found. The coding region ends with a TAA stop codon and predicts a polypeptide of 475 amino acid residues with a calculated molecular mass of 52,241 Da, which is similar to the molecular mass determined for the purified *Synechocystis* NADP$^+$-IDH subunit (44).

Comparison of the deduced amino acid sequence of the *Synechocystis icd* gene with available databases by using the FASTA program revealed that *Synechocystis* NADP$^+$-IDH is homologous to other IDHs and isopropylmalate dehydrogenases sequenced, having the highest level of amino acid identity with the *Anabaena* NADP$^+$-IDH (81% identity). The most significant difference between the bacterial NADP$^+$-IDH sequences is the presence of a 44-amino-acid-residue insertion in the cyanobacterial proteins. This extra stretch (amino acid residues 286 to 329) is conserved in the two cyanobacterial sequences available and seems to be an exclusive characteristic of NADP$^+$-IDHs from cyanobacteria. The predicted secondary structure for this region is an α-helix, located within the small α/β domain described for the *E. coli* enzyme (23), but its function in the cyanobacterial enzymes is unknown.

Disruption of the *icd* gene. In order to further investigate the role of NADP$^+$-IDH in *Synechocystis* sp. strain PCC 6803, we

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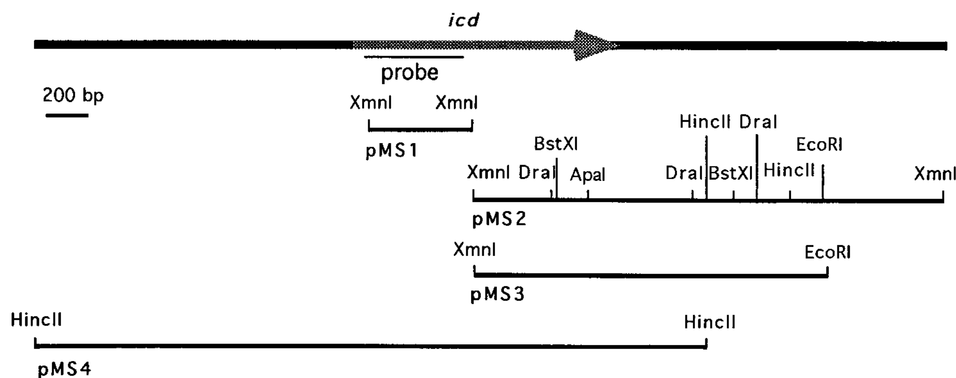

FIG. 3. Restriction map of the isolated plasmids that contain the *Synechocystis* sp. strain PCC 6803 *icd* gene. The arrow represents the predicted *icd* coding region.

tried to obtain an *icd* mutant strain. An inactivated version of the cloned gene was constructed by insertion of a chloramphenicol resistance (Cm^r) cassette. The plasmids containing the interrupted gene were introduced by transformation into *Synechocystis* sp. strain SFCΩ5, and Cm^r colonies were obtained in BG11C medium. Since a *Synechocystis icd* mutant was expected to be a glutamate auxotroph, like *icd* mutants in other bacteria (i.e., *E. coli* and *Rhizobium* sp.) (31, 37), Cm^r colonies were cultured for several rounds of segregation in the same medium supplemented with glutamate (5 mM). Analysis by Southern hybridization of Cm^r strains showed two hybridization bands: one corresponding to the wild-type fragment and another corresponding to the insertion of the 1.3-kb Cm^r cassette (data not shown). This result indicated that the gene replacement had taken place but that only some of the chromosomes contained the mutation (*Synechocystis* sp. strain PCC 6803 is a polyploid bacterium containing about 12 chromosomes per cell [29]). Addition of α-ketoglutarate to the culture medium did not increase chromosomal segregation (data not shown). These results suggested that completely segregated *Synechocystis icd* mutants were not viable, as we had previously reported for *Anabaena* sp. strain PCC 7120 *icd* mutants (45).

Modulation of *icd* transcript levels in response to nitrogen starvation. In order to determine if the increase of IDH activity and protein observed in *Synechocystis* cells under nitrogen deficiency corresponded to higher *icd* gene expression, levels of *icd* mRNA under different conditions were determined by Northern blot analysis. Total RNA from mid-exponential-phase *Synechocystis* cultures grown either with nitrate or with ammonium or after 15 h of nitrogen starvation was isolated as described in Materials and Methods and probed with a 536-bp *Xmn*I-*Xmn*I internal *icd* fragment (Fig. 3). Northern hybridization experiments revealed a smeared hybridization pattern indicating that a heterogeneous population of RNA molecules contained *icd* sequences, the largest of which was about 1.6 kb (Fig. 4A). This result suggests that *icd* transcript is monocistronic. The presence of excess 16S rRNA was probably responsible for depleting the signal in the 1.5-kb region (Fig. 4A). The level of the *icd* transcript of nitrogen-starved cells was about sevenfold higher than that of nitrate- or ammonium-growing cells (Fig. 4A). To more precisely determine the time course of *icd* transcript accumulation, nitrate-grown *Synechocystis* cells were transferred to nitrogen-free medium and samples were taken at different times for total RNA isolation. Northern blot analysis and further densitometric quantification of the autoradiograms showed that induction was observed after 1 h of nitrogen deprivation and that maximal induction was obtained after 5 h under these conditions (Fig. 5). In addition, Northern blotting was carried out to determine the level of the *icd* gene transcript from *Anabaena* sp. strain PCC 7120 cells grown with different nitrogen sources. As shown in Fig. 4B, the level of *Anabaena icd* mRNA from dinitrogen-fixing cells was about twofold higher than that of the nitrate- or ammonium-growing cells. These results suggest that *icd* gene expression was regulated by nitrogen availability in both cyanobacteria studied. On the other hand, the size of the transcript detected for *Anabaena icd* gene was about 2.0 kb (Fig. 4B). Since the size expected for a monocistronic *icd* transcript is about 1.4 kb, we cannot rule out that the *icd* gene from *Anabaena* sp. is included in a dicistronic messenger. In addition, upstream of the *Anabaena icd* gene, there are six imperfect copies of a 7-bp repeating sequence that has the consensus sequence CCCCAAT (45). Heptanucleotides with different consensus sequences have been detected near other genes in heterocystous cyanobacteria (2, 15, 21, 43, 58). The fact that these heptamer repeats are in some cases transcribed and translated, along with the lack of clear promoter sequences in the *icd* 5′ flanking region and the size of the transcript detected, could indicate that these repeats are also transcribed, the promoter of *icd* being far away from this region. Therefore, we focused on the study of the promoter of the *Synechocystis icd* gene.

Transcriptional start site mapping of the *Synechocystis* sp. strain PCC 6803 *icd* gene. Primer extension analysis was carried out to determine the transcription start point (*tsp*) of the *Synechocystis* sp. strain PCC 6803 *icd* gene. A single extension

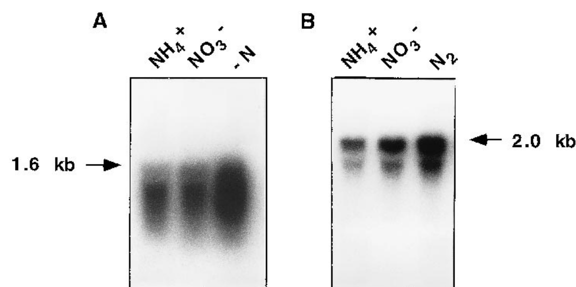

FIG. 4. Levels of *icd* transcript in *Synechocystis* sp. strain PCC 6803 and *Anabaena* sp. strain PCC 7120 cells under different nitrogen conditions. (A) Total RNA was isolated from mid-log-phase *Synechocystis* cells that used nitrate or ammonium as nitrogen source or from cells subjected to nitrogen deficiency for 15 h. RNA was denatured, electrophoresed in a 1% agarose gel, blotted, and hybridized with a 536-bp *Xmn*I-*Xmn*I *Synechocystis icd* fragment. (B) Total RNA was isolated from mid-log-phase *Anabaena* cultures that used nitrate, ammonium, or dinitrogen as nitrogen source. RNA was processed as described for panel A and probed with a 1.4-kb *Sca*I-*Acc*I *Anabaena icd* fragment. A total of 15 μg of total RNA was loaded per lane. Transcript sizes were estimated by comparison with 23S, 16S, and 5S rRNAs (42).

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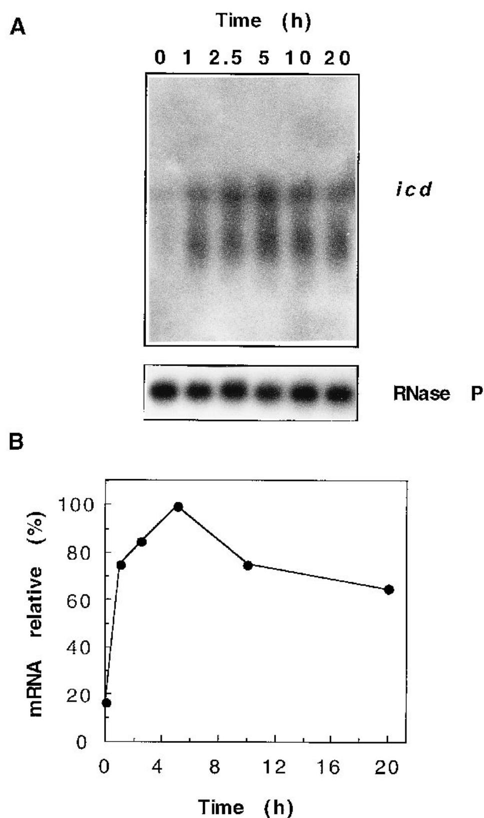

FIG. 5. Kinetics of *Synechocystis icd* transcript accumulation under nitrogen starvation. Nitrate-grown *Synechocystis* cells were harvested, washed, and transferred to nitrogen-free medium. Samples for total RNA isolation were taken at the indicated times. RNA was processed and hybridized as described for Fig. 4A. (B) The mRNA levels were quantified by densitometry, and plots were drawn of relative mRNA levels versus time. Values are averages of two hybridization experiments and are expressed as a percentage of the higher value.

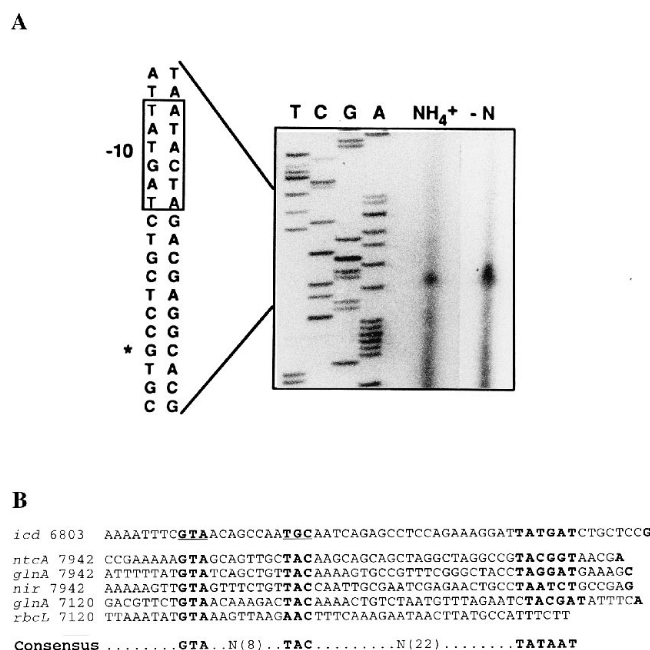

FIG. 6. (A) Primer extension analysis of the *Synechocystis icd* transcript. Total RNA (50 μg) from ammonium-grown or nitrogen-starved cells was annealed to an oligonucleotide of the *icd* gene and extended with avian myeloblastosis virus reverse transcriptase as described in Materials and Methods. Lanes T, C, G, and A contain a dideoxy sequencing ladder carried out with the same primer. The transcription start nucleotide is indicated by an asterisk. (B) Alignment of *Synechocystis icd* promoter region with several NtcA-regulated promoters from different cyanobacteria. The NtcA binding site, the −10 box, and transcription start points are in boldface.

product was detected in extension experiments using total RNA from either ammonium-grown or nitrogen-starved *Synechocystis* cells (Fig. 6). The transcription start point was localized to nucleotide −27 with respect to the first translated nucleotide. The product of the extension reaction was much more abundant with RNA from nitrogen-starved cells than with RNA isolated from ammonium-grown cells (Fig. 6A). A sequence with five of six matching the −10 σ^{70}-dependent *E. coli*-like promoter-consensus sequence occurred upstream of the *icd tsp* (TATGAT). However, no obvious −35 consensus sequence was observed. The DNA sequence that precedes the *tsp* of the *icd* gene was compared with those of previously known nitrogen-regulated promoters from cyanobacteria. A sequence (GTAN$_8$TGC) exhibiting near-perfect identity with the consensus binding site of the transcription factor NtcA (GTAN$_8$TAC) (34) was detected 22 bases upstream of the −10 sequence (Fig. 6B). It has been proposed that nitrogen- and NtcA-regulated promoters consist of a −10 sequence similar to the *E. coli* consensus and an NtcA-binding site, at position −39.5 or −40.5 with respect to the *tsp*, that substitutes for the typical −35 hexamer (Fig. 6B) (34). Therefore, the structure of the *icd* gene promoter is very similar to those of the NtcA-regulated promoters.

Binding of NtcA to the promoter of the *Synechocystis* sp. strain PCC 6803 *icd* gene. In order to test whether the transcription activator NtcA binds to the promoter of the *Synechocystis icd* gene, we have performed mobility shift experiments with the purified *Synechocystis* NtcA protein. The *ntcA* gene from *Synechocystis* strain PCC 6803 was cloned by means of standard PCR techniques and introduced in the pGEX-4T-1 expression vector. The GST-NtcA fusion protein was purified by affinity chromatography on glutathione agarose. One single band of about 50 kDa (fusion protein between GST [27.5 kDa] and NtcA [25.0 kDa]) and no degradation products were visible after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining. As shown in Fig. 7, purified NtcA retarded a *Spe*I-*Xmn*I 128-bp fragment that contains the *icd* promoter (from −100 to +28 with respect to the *tsp*). A 30-fold excess of unlabeled fragment significantly reduced the amount of labeled NtcA-*icd* promoter complex. As a control, GST protein expressed with the same vector and purified by the same protocol was unable to retard the fragment containing the *icd* gene promoter (Fig. 7). These data

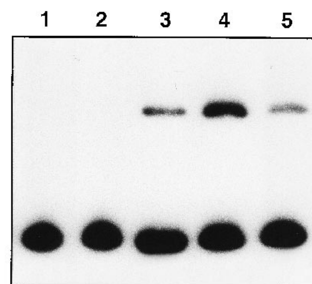

FIG. 7. Gel retardation analysis of the binding of NtcA to a *Synechocystis icd* promoter fragment. A 128-bp DNA fragment encompassing the *icd* promoter was incubated in the presence of various concentrations of purified NtcA or GST protein. Lane 1, no protein; lane 2, 5 μM GST; lane 3, 0.25 μM NtcA; lane 4, 0.5 μM NtcA; lane 5, 0.5 μM NtcA plus a 30-fold excess of unlabeled probe.

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indicate that the NtcA protein specifically binds to the *Synechocystis* sp. strain PCC 6803 *icd* regulatory region.

DISCUSSION

In cyanobacteria, NADP^{+}-IDH is generally considered to be the enzyme responsible for supplying the carbon skeletons (α-ketoglutarate) needed for ammonium assimilation; therefore, regulation of NADP^{+}-IDH in response to changes in nitrogen availability could be expected. In fact, in *Synechocystis* sp. strain PCC 6803, the level of NADP^{+}-IDH enzyme activity and the amount of NADP^{+}-IDH protein and the *icd* transcript increase between five- and sevenfold during nitrogen starvation (Fig. 1, 2, 4, and 5). This fact agrees with the strong increase in the intracellular concentration of α-ketoglutarate detected 4 h after transferring nitrate-grown *Synechocystis* cells to nitrogen-free medium (sevenfold) (40). However, in nitrogen-starved cells the action of transaminases may also account for a considerable fraction of the α-ketoglutarate increase.

In the case of *Anabaena* sp., expression of the *icd* gene was maximal under dinitrogen-fixing conditions (Fig. 2 and 4), correlating with the higher *icd* expression in *Synechocystis* sp. under nitrogen starvation, since dinitrogen fixation is the most nitrogen-limiting condition for *Anabaena* sp. We have previously reported that a partially segregated *icd* mutant from *Anabaena* sp. is unable to grow on nitrogen-free medium without α-ketoglutarate added (45). These results are in concordance with the hypothesis that the requirement of α-ketoglutarate must be higher in this medium, probably because ammonium assimilation is restricted to heterocysts under these conditions.

Since α-ketoglutarate is the substrate for glutamate synthesis, regulation of NADP^{+}-IDH must be coordinated with that of the GS-GOGAT pathway. In fact, expression of the *glnA* gene (structural gene for glutamine synthetase type I) has been also shown to be regulated in response to nitrogen starvation in several cyanobacteria. Transcript from the *Synechococcus* sp. strain PCC 7002 *glnA* gene increases three- to fivefold when the cells are starved of nitrogen (59) while, in strain PCC 7942, a strong increase in the amount of *glnA* mRNA has been described after transferring ammonium-grown cells to nitrate-containing or nitrogen-free medium (9, 34). In *Anabaena* sp. strain PCC 7120, *glnA* is transcribed from multiple promoters that are differentially expressed in response to changes in the nitrogen source, the highest expression being under dinitrogen-fixing conditions (56). The amount of *glnA* transcript from *Synechocystis* sp. strain PCC 6803 also increases (two- to fourfold) under nitrogen deficiency (49a). The increase of α-ketoglutarate mediated by the induction of NADP^{+}-IDH during nitrogen starvation, together with the induction of GS expression, may correspond to a metabolic mechanism to guarantee the immediate assimilation of the available nitrogen through the GS-GOGAT pathway. In addition, α-ketoglutarate is not only the substrate for ammonium assimilation but also a very important regulatory metabolite involved in the regulation of nitrogen assimilation pathways in cyanobacteria (16, 40) and other prokaryotes (36). This regulatory role could explain why it was impossible to obtain an *icd* null mutant in *Synechocystis* strain PCC 6803 as well as in *Anabaena* strain PCC 7120 (45).

The NtcA protein is a positive regulator of genes subjected to nitrogen control in cyanobacteria. NtcA belongs to the family of bacterial DNA-binding proteins of which cyclic AMP receptor protein is the prototype (28, 57). It has been demonstrated that NtcA (BifA) binds directly to the promoter regions of *glnA*, *rbcL*, *xisA*, and *nifH* genes in *Anabaena* sp. strain PCC 7120 (47, 60) and to the promoter regions of *glnA*, *ntcA*, and the *nirAnrtABCDnarB* operon in *Synechococcus* sp. strain PCC 7942 (34). Primer extension experiments showed that the structure of the *Synechocystis* sp. strain PCC 6803 *icd* gene promoter agrees with that of an NtcA-regulated promoter (Fig. 6B). The putative NtcA binding sequence from the *Synechocystis icd* promoter (GTAN$_8$TGC) contains only one mismatch with respect to the defined consensus (GTAN$_8$TAC) and presents also the A:T-rich region found upstream of all the NtcA binding sites so far described (Fig. 6B). In fact, purified *Synechocystis* strain PCC 6803 NtcA protein is able to bind specifically to a short DNA fragment containing the *icd* promoter (Fig. 7). All these data together with the expression pattern of the *icd* gene strongly suggest that transcription of the *Synechocystis icd* gene is positively regulated by NtcA.

NtcA induces transcription from several promoters in the absence of ammonium, under conditions of nitrate utilization (34). All these promoters (p*glnA*, p*ntcA*, and p*nirA* from *Synechocystis* sp. strain PCC 7942) contain an NtcA binding site that matches exactly the consensus sequence GTAN$_8$TAC. The fact that the NtcA binding site of the *icd* promoter does not exactly fit the consensus motif could explain why transcription of the *icd* gene does not increase in nitrate-grown cells compared with ammonium-grown cells. Since nitrogen starvation is a more ammonium-deficient condition than nitrate utilization, and since *ntcA* gene transcription is autoregulated (34), levels of NtcA protein may be higher under nitrogen deficiency than under nitrogen assimilation. Activation of the *icd* promoter, with a low-affinity binding site, would require a higher concentration of NtcA protein that would be reached in nitrogen-starved cells but not in nitrate-growing cells. A similar situation is found in the promoter of the *Anabaena* sp. strain PCC 7120 *xisA* gene, which contains three nonconsensus NtcA binding sites. *xisA* encodes a site-specific recombinase required for the rearrangement of *nif* genes and is induced only under nitrogen starvation, not under nitrate assimilation (47, 60).

In summary, the regulation of the cyanobacterial *icd* gene in response to nitrogen availability indicates a coordinate expression of the genes involved in carbon skeleton supply and those of nitrogen assimilation.

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