Nitrogen-regulated Genes for the Metabolism of Cyanophycin, a Bacterial Nitrogen Reserve Polymer

EXPRESSION AND MUTATIONAL ANALYSIS OF TWO CYANOPHycin SYNTHETASE AND CYANOPHycinASE GENE CLUSTERS IN THE HETEROCYST-FORMING CYANOBACTERIUM ANABAena SP. PCC 7120*

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Two gene clusters each encoding the cyanophycin-metabolism enzymes cyanophycin synthetase and cyanophycinase are found in the heterocyst-forming cyanobacterium Anabaena sp. PCC 7120. In cluster cph1, the genes cphB1 and cphA1 were expressed in media containing ammonium, nitrate, or N₂ as nitrogen sources, but expression was higher in the absence of combined nitrogen taking place both in vegetative cells and heterocysts. Both genes were cotranscribed from three putative promoters located upstream of cphB1, and, additionally, the cphA1 gene was expressed monocistronically from at least two promoters located in the intergenic cphB1-cphA1 region. Both constitutive promoters and promoters dependent on the global nitrogen control transcriptional regulator NtcA were identified. In cluster cph2, the cphB2 and cphA2 genes, which are found in opposite orientations, were expressed as monocistronic messages in media containing ammonium, nitrate, or N₂, but expression was higher in the absence of ammonium. Expression of the cph2 genes was lower than that of cph1 genes. Analysis of cph gene insertion mutants indicated that cluster cph1 genes contributed more than cluster cph2 genes to cyanophycin accumulation in the whole filament as well as in heterocysts. Diazotrophic growth was more severely impaired in cyanophycinase than in cyanophycin synthetase mutants, indicating that cyanophycin, although normally synthesized in the heterocysts, is not required for heterocyst function and that the inability to degrade this polymer is detrimental for the diazotrophic growth of the cyanobacterium.

Cyanobacteria are oxygenic photoautotrophs that make an important contribution to primary productivity in our planet. They fix CO₂ through the reductive pentose phosphate pathway and preferentially assimilate inorganic sources of nitrogen. Many cyanobacteria are able to carry out the fixation of atmospheric nitrogen, and, as a way to protect the nitrogen fixation machinery from oxygen, some filamentous strains differentiate cells called heterocysts where nitrogenase is con

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cyanophycinase has been identified in the genomic sequence of Synechocystis sp. strain PCC 6803 (12), and ORFs \(^2\) of both Synechocystis sp. and Anabaena sp. have been described to encode plant-type asparaginases able to hydrolyze \(\beta\)-Asp-Arg bonds and that, thus, may be responsible for the last step of cyanophycin degradation (13). The cphB and cpha genes have also been identified in whole genome sequence projects of several cyanobacteria.

Putative cyanophycin synthetase genes have recently been identified in the genomic sequences of a number of eubacteria different from cyanobacteria, catalyzing a very specific degradation of cyanophycin to \(\beta\)-Asp-Arg dipeptides (16). Thus, cyanophycin appears to occur in a wide range of bacteria that make use of diverse metabolic options including phototrophy, anaerobic respiration, fermentation, and chemolithoautotrophy, where it can serve, perhaps among other functions, as a nitrogen reserve or an external carbon source. Therefore, cyanophycin seems to have a role in the biology of bacteria much wider than previously recognized.

The present work deals with the genetic systems for cyanophycin synthesis and degradation in the filamentous, heterocyst-forming cyanobacterium Anabaena sp. PCC 7120, which is amenable to genetic manipulation and whose entire genomic sequence is available (17). The regulation of the expression of cyanophycin metabolism genes with regard to the nitrogen regime and cell type in the diazotrophic filament has been studied, and the involvement of cyanophycin in diazotropic metabolism has been investigated through the generation of cyanophycin metabolism gene mutants.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—This study was carried out with the heterocyst forming cyanobacterium Anabaena sp. PCC 7120 and an insertional mutant of the \(n\)tcA gene, strain CSE2 (18). Anabaena sp. PCC 7120 was grown axenically in BG11 medium, which contains 17.6 mM NaNO\(_3\), (19), in BG11, (nitrogen-free) medium or in BG11 medium supplemented with 8 mM NH\(_4\)Cl and 16 mM TES-NaOH buffer (pH 7.5). Strain CSE2 was grown in ammonium-containing medium in the presence of 2 \(\mu\)g ml\(^{-1}\) of streptomycin and 2 \(\mu\)g ml\(^{-1}\) of spectomycin. For plates, the medium was solidified with 1% separately autoclaved agar (Difco). Liquid cultures were incubated at 30 °C in the light (75 microeinsteins \(m^{-2}\) s\(^{-1}\)) with shaking (80–90 rpm). Anabaena mutants carrying gene cassette C.K3 (20) were routinely grown in medium supplemented with 5 \(\mu\)g ml\(^{-1}\) Em. Growth rates were estimated from the increase in protein concentration of the cultures, determined by a modified Lowry procedure (23) in 0.2-ml aliquots periodically withdrawn from the cultures. The growth rate constant (\(\mu\)) corresponded to the doubling time (\(t_d\), where \(t_d\) represents the doubling time).

**Mutant Construction**—To inactivate gene cphA1, a 1.63-kb DNA fragment from the cphA1 region of Anabaena sp. PCC 7120 was amplified by PCR using oligonucleotides CB1–1 (corresponding to nucleotides +3 to +43 relative to the transcription start of cphA1) and CA1–3 (corresponding to nucleotides +536 to +636 relative to the translation start of cphA1) and ended with a XhoI restriction site). PCR products were cloned in vector pGM-E to generate plasmid pCSS35. The Em\(^{\text{R}}\)/Cm\(^{\text{R}}\) gene cassette excised with HindIII (24) and filled in with Klenow enzyme, rendering plasmid pCSS34. The SalI-NcoI fragment from pCSS34, filled in with Klenow enzyme, was ligated to the sacB-containing vector pRL27 (22) digested with NruI, rendering plasmid pCSS35.

To inactivate gene cphA2, a 1.7-kb DNA fragment from the cphA2 region of Anabaena sp. PCC 7120 was amplified by PCR using oligonucleotides CB2–1 (corresponding to nucleotides +1654 to +1763 with respect to the translation start of cphA2 and ended with a XhoI restriction site) and CA2–3-XhoI (corresponding to nucleotides +60 to +77 relative to the translation start of cphA2 and ended with a XhoI restriction site). PCR products were cloned in vector pGM-E to generate plasmid pCSS33. The Em\(^{\text{R}}\)/Cm\(^{\text{R}}\) gene cassette excised with HindIII was inserted into the HindIII site that is present in the Anabaena DNA insert of pCSS50, rendering plasmid pCSS31. The PvuII fragment from pCSS31 was ligated to the sacB-containing vector pRL27 (22), digested with BglIII, and filled in with Klenow enzyme, rendering plasmid pCSS32.

To inactivate gene cphB2, a 1.7-kb DNA fragment from the cphB2 region of Anabaena sp. PCC 7120 was amplified by PCR using oligonucleotides CB2–1 (corresponding to nucleotides +1654 to +1763 with respect to the translation start of cphB2 and ended with a XhoI restriction site) and CA2–3-XhoI (corresponding to nucleotides +60 to +77 relative to the translation start of cphA2 and ended with a XhoI restriction site). PCR products were cloned in vector pGM-E to generate plasmid pCSS33. The Em\(^{\text{R}}\)/Cm\(^{\text{R}}\) gene cassette containing erm and cat genes excised with XbaI from plasmid pCSS52, which contains the SalI-XbaI fragment from pRL271 cloned in vector pC20R, and filled in with Klenow enzyme was inserted into the Eco47III site that is present in the Anabaena DNA insert of pCSS33, rendering plasmid pCSS34. The XhoI fragment from pCSS34 was ligated to the sacB-containing vector pRL278 digested with XhoI, rendering plasmid pCSS35.

Constructs generated in vitro bearing a gene cassette inserted into cphA1, cphA2, cphB1, or cphB2 and cloned in sacB-containing vectors were transferred by conjugation (26) to Anabaena sp. to generate strains bearing mutations in the cphA1 and cphB2 genomic regions. For generation of strains CSS7, CSS13, CSS21, and CSS25, E. coli strain HB101 containing plasmid pCSS35, pCSS39, pCSS52, or pCSS55 and helper plasmids pRL528 (28) and pRL501-W45 (27) or pRL232 (28) was mixed with E. coli ED854 carrying the conjugative plasmid pL443 and thereafter with Anabaena sp. For the generation of double mutants, plasmids pCSS39 and pCSS55 were transferred to strain CSS7 to generate strains CSS35 and CSS27, respectively, plasmid pCSS52 was transferred to strain CSS13 to generate strain CSS23, and plasmid pCSS55 was transferred to strain CSS21 to generate strain CSS36. E. coli strains were isolated (26), and double recombinants were identified as clones resistant to the antibiotic for which resistance was encoded in the inserted gene cassette, resistant to sucrose, and sensitive to the antibiotic for which the resistance determinant was present in the vector portion of the transferred plasmid and were confirmed by PCR analysis. Homozygous mutant clones were selected for this study.

**Labeling and Analysis of DNA**—Total DNA from Anabaena sp. PCC 7120 and its derivatives was isolated as previously described (25). For sequencing ladders used in primer extension analysis, sequencing was carried out by the dideoxy chain termination method, using a T7-Sequencing\(^{\text{TM}}\) kit (Amersham Biosciences) and (\(\alpha\)-\(^{35}\)S)-dATP. DNA fragments were purified from agarose gels with the Geneclean II kit (Bio 101). Southern blot analysis, plasmid isolation from E. coli, transformation of E. coli, digestion of DNA with restriction endonucleases, ligation with T4 ligase, and PCR were performed by standard procedures (29).
mobility shift assays were obtained by PCR amplification. Oligonucleotides CB1–4 (corresponding to positions −289 to −679 relative to the translation start of cphB1), CB1–5 (complementary to positions −250 to −272 relative to the translation start of cphB1), CB1–7 (complementary to positions −458 to −477 relative to the translation start of cphB1), and CB1–9 (corresponding to positions −485 to −465 relative to the translation start of cphB1) and plasmid pCSS88, containing the cphB1 promoter sequence (cloned by PCR with oligonucleotides CB1–4 and CB1–5 in vector pGEM-T), were used to obtain DNA fragments of the cphB1 upstream region. Oligonucleotides CA1–6 (corresponding to positions −214 to −196 relative to the translation start of cphA1) and CA1–5 (complementary to positions −16 to −3 relative to the translation start of cphA1) and plasmid pCSS87 as a template were used for PCR amplification of the cphA1 upstream region. For competition bands, a DNA fragment from the region upstream from glnA containing an NtcA-binding site (18) was used as a specific binding fragment, and a DNA fragment of the pBluescript vector was used as a nonspecific fragment. In the case of the glnA upstream region, oligonucleotides GA3 (corresponding to positions −238 to −215 relative to the translation start of glnA) and GA4 (complementary to positions −70 to −87 relative to the translation start of glnA) and plasmid pAN503 (30) were used for PCR. In the case of the pBluescript DNA fragment, oligonucleotides Forward and Reverse were used, rendering a DNA fragment of 220 nucleotides. DNA fragments were end-labeled with T4 polynucleotide kinase (Roche Applied Science) and [γ-32P]ATP as described previously (29). Assays were carried out as described previously (31) with 1 μmol of labeled fragment and 0.1–3 μmol of NtcA purified from E. coli strain DH5α (pCSAM61) bearing the strain PCC 7120 ntcA gene cloned in pTRc99A vector (Amersham Biosciences) and thus expressed from the trc promoter. Images of radioactive gels were obtained using a Cyclone storage phosphor system (Packard).

RNA Isolation and Analysis—Cells used for RNA isolation were exponentially growing in the light (75 microeinsteins m−2 s−1) in liquid BG11 or BG11a media (19) or in medium BG11 containing 8 mM NH4Cl and 16 mM Tris-NaOH buffer (pH 7.5), supplemented with 10 mM of NaHCO3 and bubbled with air and CO2 (1%, v/v). Alternatively, filaments growing in ammonium-containing medium were harvested at room temperature and either used directly or washed with and resuspended in BG11a medium and further incubated under culture conditions for the number of hours indicated in each experiment. For the isolation of RNA from heterocysts, cells were grown in ammonium-containing medium until they reached a chlorophyll a concentration of 3–5 μg ml−1. Filaments were then washed with and resuspended in nitrogen-free medium (BG11a) and further incubated until mature heterocysts were observed (19 h). Heterocysts were then isolated as described (32). Total RNA from whole filaments or from isolated heterocysts was isolated in the presence of ribonucleoside-vanadyl complex as previously described (33). For Northern analysis, 40–70 μg of RNA were loaded on a lane and subjected to electrophoresis in 1% agarose denaturing formaldehyde gels. Transfer and fixation to Hybond-N+ membranes (Amersham Biosciences) were carried out using 0.1 μm NaOH. Hybridization was performed at 65°C according to the recommendations of the manufacturer of the membranes.

The cph probes were internal fragments of these genes amplified by PCR, using plasmid pCSS3 as a template and oligonucleotides CA1–1 and CA1–2 (see above) in the case of the cphA1 probe; plasmid pCSS7 as a template and oligonucleotides CB1–1 (see above) and CB1–2 (complementary to nucleotides +885 to +864 with respect to the translation start of cphB1) for the cphB1 probe; plasmid pCSS3 as a template and oligonucleotides CA2–2 and CA2–3 (see above) for the cphA2 probe; and plasmid pCSS50 as a template and oligonucleotides CA1–3 and oligonucleotides CB2–3 (corresponding to nucleotides +558 to +537 with respect to the translation start of cphB2) for the cphB2 probe. All probes were [32P]-labeled with a Ready to use DNA labeling kit (Amersham Biosciences) using [γ-32P]ATP. Images of radioactive filters were obtained with a Cyclone storage phosphor system and OptiQuant image analysis software (Packard). The rnpB gene, which encodes a stable RNA (34), was used as an RNA loading and transfer control.

Primer extension analysis of the cph transcripts was carried out as previously described (35) with [32P]-labeled oligonucleotides CB1–6 (corresponding to nucleotides −188 to −220 with respect to the translation start of cphB1) and CB1–5 (see above) for the case of cphB1 and CA1–4 (complementary to nucleotides +74 to +55 with respect to the translation start of cphA1) and CA1–5 (see above) for the case of cphA1. Cyanophycin Measurements—Cells subjected to a treatment to force cyanophycin accumulation were used. Cells of 150-ml liquid cultures in BG11 medium supplemented with 10 mM NaHCO3 and bubbled with air and CO2 (1%) were harvested in the exponential growth phase (2–5 μg of Chl ml−1), washed twice with BG11a medium, and used to inoculate 150-ml cultures in BG11a medium supplemented with bicarbonate and bubbled with air and CO2 (1%). These cultures were then incubated for about 8 h under culture conditions. After this incubation, NH4NO3 was added at 4 mM final concentration, and the cultures were incubated under dim light (5 microeinsteins m−2 s−1) for 12–14 h. Cells from 100 ml of these cultures were collected at room temperature, washed twice with, and resuspended in, milliQ-purified and autoclaved H2O, and disrupted with a French Press (twice at 20,000 p.s.i.). After measuring the obtained volume of cell extract, chlorophyll a was determined in a 100-μl sample. The remnant of each extract was centrifuged for 15 min at 15,000 rpm in a SS34 rotor, the resulting supernatants were discarded, and the pellets were washed twice with 11 ml of milliQ-purified, autoclaved H2O and resuspended in 1 ml of 0.1 M HCl. After 2–4 h of incubation at room temperature and centrifugation under the same conditions, the resulting supernatants were stored. The pellets were resuspended in 1 ml of 0.1 M HCl, incubated overnight at room temperature, and again subjected to centrifugation. The obtained supernatants were combined with those obtained after the first centrifugation, stored at 4°C, for no longer than 1 week, and used for arginine determination, which was carried out by the Sakaguchi method as modified by Messineo (36).

Microscopy—Cells grown during 7–10 days in shaken BG11a liquid medium were observed and photographed with a Zeiss Axioskop microscope equipped with an MC 80 camera.

RESULTS

Identification of Two Clusters of Cyanophycin Metabolism Genes—In the genomic sequence of Anabaena sp. PCC 7120 (17), two clusters of ORFs showing homology to cyanophycin synthetase or cyanophycinase-encoding genes could be identified (Fig. 1). We have named cluster 1 to that containing genes more similar to those identified in other cyanobacteria (e.g. see Refs. 10, 12, and 37). Gene cphB1 would encode a cyanophycinase of 298 amino acids showing 99% identity to CphB from A. variabilis (12) and 60% identity in a 269-amino acid overlap to cyanophycinase from Synechocystis sp. PCC 6803 (37). Gene cphA1 would encode a cyanophycin synthetase of 901 amino acids.

Fig. 1. Schematic representation of two gene clusters found in the genome of Anabaena sp. PCC 7120 that contain genes homologous to cph genes (drawn from data available on the World Wide Web at www.kazusa.or.jp/cyanobase/Anabaena/). The ORF name, length in bp, and proposed gene name are indicated. Flanking ORFs are also indicated. Double lines indicate probes used for Northern analysis (see "Experimental Procedures"). The insertion sites of the C3, C5, C13, or Em3′ gene cassettes to generate mutants of each gene are shown (see "Experimental Procedures" for details).
an acids showing 99% identity to the product of cpha from A. variabilis (10) and 70% identity in an 870-amino acid overlap to Cpha from Synechocystis sp. PCC 6803 (37). In Anabaena sp. PCC 7120, cphB is located upstream from cpha, and both genes show the same orientation (Fig. 1).

Gene cphB2 would encode a 293-amino acid product showing 43% identity in a 259-amino acid overlap to cyanophycinase from A. variabilis and 40% (248-amino acid overlap) to CphB from Synechocystis sp. PCC 6803. Gene cpha2 from Anabaena sp. PCC 7120 would encode a 636-amino acid product showing 33% identity in a 423-amino acid overlap to Cpha from strain PCC 6803 and 30% (421-amino acid overlap) to the protein from A. variabilis. In this gene cluster of strain PCC 7120, the cphB2 and cpha2 genes are arranged in opposite orientations separated by 914 bp that include an ORF named ast0572 that would encode a 75-amino acid product showing no significant similarity to any protein in the data bases (Fig. 1).

Expression of cphB1 and cpha1—When expression of the cpha1 gene was analyzed by RNA/DNA hybridization, transcripts of up to ~5 kb could be detected that were present at higher levels in cultures growing diazotrophically than in those growing with combined nitrogen, either nitrate or ammonium (Fig. 2a). Because the length from the beginning of cphB1 to the end of cpha1 is 3,774 bp, the ~5-kb transcript could encompass the message of the two genes. Expression of cphB1 was also tested with RNA from isolated heterocysts, with which only hybridization signals corresponding to smaller transcripts could be detected (Fig. 2a).

Northern experiments performed with a probe of the cpha1 gene also showed hybridization to transcripts of up to ~5 kb in size (Fig. 2b), similar to those detected with the cphB1 probe (Fig. 2a). Hybridization signals were observed with RNA isolated from whole filaments grown under different conditions of nitrogen nutrition or from isolated heterocysts but were more abundant in the diazotrophic culture. Transcripts of ~3 kb were also detected with the cpha1 gene probe (Figs. 2, b and c) that could correspond to cpha1 monocistronic transcripts. To test this possibility, cpha1 was expressed in analysis by strain CSS13, an insertional mutant that bears gene cassette C.K3 inserted into the cpha1 gene in the orientation opposite to that of the gene (see below and “Experimental Procedures” for details). Fig. 2c shows that only hybridization signals corresponding to ~3 kb and smaller could be detected in this mutant strain confirming independent transcription of the cpha1 gene.

Primer extension experiments with two different primers (see “Experimental Procedures”) detected three 5′ transcript ends upstream of cphB1 (Fig. 3a and not shown). The first 5′ end, Iα, in Fig. 3a, was located at nucleotide −339 with respect to the cphB1 initiation codon, showed low relative abundance being preferentially detected with RNA from diazotrophic whole filaments and from isolated heterocysts, and its level increased upon combined nitrogen deprivation (see Fig. 4c).

The second 5′ end (Iβ) was located at nucleotide −357 and appeared to be the most abundant in whole filaments, being detected at similar levels irrespective of the nitrogen source used for growth. Although at a lower level, it was also present in heterocyst RNA. Finally, 5′ end IIIα, corresponding to nucleotide −499, was preferentially found with RNA from diazotrophic cultures and was also present in heterocyst RNA. Its cellular level also increased upon combined nitrogen deprivation (see Fig. 4c). Although some other putative 5′ transcript ends are observed in Fig. 3a, only those above mentioned were repeatedly detected with different oligonucleotides.

To test for the existence of possible functional promoters upstream from cpha1, primer extension experiments were performed with two different primers of the 5′ region of this gene (Fig. 3b and not shown) using RNA from both the wild type and the cphB1 mutant strain CSS13. For the wild type, five different 5′ RNA ends were detected corresponding to positions −89 (Iα), −116 (IIα), −141 (IIIα), −191 (IVα), and −230 (Vα) with respect to the cpha1 initiation codon. 5′ RNA ends IVα and Vα corresponded to nucleotides inside the cpha1 gene and were not detected in RNA from the cphB1 mutant; the corresponding RNA molecules could represent degradation products of transcripts initiated at promoters upstream from cphB1. 5′ ends Iα, IIα, and IIIα corresponded to positions located in the intergenic cphB1-cpha1 region. 5′ ends Iα and IIIα were detected with RNA from both the wild type and the cphB1 mutant strain (Fig. 3b, a and c), and they were observed with similar intensity with RNA isolated from wild-type cells subjected to any tested nitrogen regime but were only barely detected with RNA from isolated heterocysts (Fig. 3b). On the other hand, 5′ end IIα was detected with RNA from the wild type and, although more weakly, also with RNA from the cphB1 mutant (Fig. 3c), was specially represented in RNA isolated from diazotrophic cultures, and was also present in RNA from isolated wild-type heterocysts, where it was the more abundant species (Fig. 3b).

Thus, 5′ RNA ends IIIα and IIα (and possibly also Iα) could represent true tsps of the cpha1 gene located in the intergenic region between cphB1 and cpha1. The distance from the IIIα
and IIA positions to the end of cphA1 is 2,846 and 2,821 bp, respectively, conforming to the /H11011 3-kb transcript size (Fig. 2c).

NtcA-dependent Expression of cphB1 and cphA1—Expression of the Anabaena cphB1 and cphA1 genes was tested by Northern and primer extension analysis in an insertional mutant of ntcA, strain CSE2 (18). Whereas, as described above, an increase in the levels of cphB1 (Fig. 4a) and cphA1 (Fig. 4b) transcripts could be observed in the wild-type strain when the...
cells were subjected to combined nitrogen deprivation, this increase was somewhat lower in the ntcA mutant. Regarding the 5' transcript ends upstream of cphB1, whereas abundance of IIa was similar in the wild type and in strain CSE2, induction of neither Ia nor IIIa took place in this mutant (Fig. 4c). These results indicate a role of NtcA in the activation of expression of the two putative nitrogen-regulated promoters located upstream of cphB1. For the 5' ends upstream of cphA1, the abundance of Ia, IIa, and IIIa in both the wild type and the ntcA mutant did not change significantly after 24 h of incubation in the absence of combined nitrogen (Fig. 4d). (Because strain CSE2 does not develop heterocysts, NtcA dependence of cph gene expression in these differentiated cells could not be assessed.)

To further analyze the basis for the dependence on NtcA of expression of cph1 genes, binding of NtcA to the DNA region upstream of cphB1 and to the cphB1-cphA1 intergenic region was tested. Mobility shift of a DNA fragment covering the region from −263 to −693 of the cphB1 gene (Fig. 5a) was specifically promoted by NtcA, producing two retarded bands (Fig. 5b). When any of two DNA fragments covering positions −250 to −477 (fragment B; Fig. 5c) and −458 to −697 (fragment C; Fig. 5d), respectively, were used, single retarded bands were observed in each case that were competed by an excess of an unlabeled DNA fragment containing a well-characterized NtcA binding site but not by an excess of a DNA fragment containing no NtcA binding site. These results indicated that NtcA specifically binds to two different sites in the promoter region of cphB1. The dependence of NtcA binding on the concentration of NtcA showed that NtcA affinity for binding to fragment C (Kd = 1.77 μM) is higher than that for fragment B. Additionally, footprinting experiments (not shown) revealed that NtcA changed the sensitivity to DNase I degradation of a DNA stretch encompassing positions −380 to −625, thus confirming the presence in it of NtcA binding site(s).

Mobility shift assays were also performed with a DNA fragment encompassing sequences from +16 to −214 of cphA1, showing specific binding of NtcA to this DNA region at a single site (Kd = 0.37 μM) (Fig. 6).

Expression of cphB2 and cphA2—Expression of cphB2 and cphA2 was tested by Northern analysis (Fig. 7). When the cphB2 probe was used, a hybridization band corresponding to a transcript of −0.9 kb was detected (Fig. 7a), and total hybridization signals were more abundant with RNA from cultures maintained in the absence of ammonium. A 0.9-kb mRNA would correspond to a monocistronic transcript of the cphB2 gene that is 881 bp long. The cphA2 probe showed a transcript of −2.1 kb that was also more abundant in the absence than in the presence of ammonium (Fig. 7b). Cellular levels of this transcript were observed to increase when ammonium-grown cultures were subjected to combined nitrogen deprivation (Fig. 7c). A 2.1-kb mRNA would correspond to a monocistronic transcript of the cphA2 gene that is 1,910 bp long. It should be noted that hybridization to the cphB2 gene probe was difficult to detect, probably reflecting a lower expression level of the cphB2 than of the cphA2 gene.

Mutant Strains Bearing Inactivated cph Genes—To analyze the involvement of each cph gene cluster in the accumulation of cyanophycin in Anabaena filaments as well as the putative role of cyanophycin in diazotrophic growth, mutant strains that carry inactivated versions of cph genes were constructed. In vitro generated constructs including antibiotic resistance-encoding gene cassettes inserted into a cph gene or gene fragment were transferred to strain PCC 7120. The mutagenic constructs were inserted into the Anabaena genome by double recombination, and clones bearing only the mutant version of the transferred gene were selected in each case (see “Experimental Procedures” for details). Single mutants were generated that bear an inactivated cphB1 (strain CSS13), cphA1 (strain CSS7), cphB2 (strain CSS21), or cphA2 (strain CSS25) gene (Table 1).
Double mutants cphA1 cphB1 (strain CSS35), cphA2 cphB2 (strain CSS36), cphA1 cphA2 (strain CSS27), and cphB1 cphB2 (strain CSS23) were also generated.

Cyanophycin accumulation was tested in cells of each of those mutants grown on nitrate, subjected to an 8-h period of combined nitrogen deprivation, and finally incubated overnight under low light with ammonium nitrate (see “Experimental Procedures”). Results obtained are shown in Table I. Mutation of cphA1 rendered a strain (CSS7) with negligible levels of cyanophycin, similar to those of the negative control Synechococcus sp. PCC 7942, which does not synthesize cyanophycin (38). In contrast, mutation of the cphA2 gene had only a minor effect on cyanophycin accumulation (cyanophycin content in strain CSS25 was ~78% that of the wild type). Cyanophycin content in the double mutant cphA1 cphA2 (strain CSS27) was similar to that of the single mutant cphA1. It can be concluded that, under our experimental conditions, CphA1 contributes the bulk of total cyanophycin synthesis in strain PCC 7120, although CphA2 also produces an active synthetase.

Mutation of cphB1 (see strain CSS13) rendered cyanophycin accumulation at levels ~2.4-fold those of the wild-type strain. Mutation of cphB2 had little effect on cyanophycin accumulation (levels in strain CSS21 were ~1.3-fold those of the wild type). Cyanophycin accumulation levels in the double mutant cphB1 cphB2 (strain CSS23) were similar to those of the single mutant cphB1. Thus, CphB1 contribution to total cyanophycin degradation under our experimental conditions was much bigger than that of CphB2. The double mutant of cluster 1, strain CSS35, showed cyanophycin levels ~22% higher than those of the cphA1 mutant, and the double mutant of cluster 2, strain CSS36, had a cyanophycin content ~12% lower than that of the wild-type strain. These results confirm a low contribution of cphA2 to total cyanophycin synthesis in strain PCC 7120.

The ability of cph mutants to grow diazotrophically was also investigated (Table I). Whereas the strains mutated only in cyanophycin synthetase genes showed growth rates on N2 that were only slightly lower than those of strain PCC 7120 (percentage of the wild-type growth rate constant was 90, 94, and 86% for the cphA1, the cphA2 and the cphA1 cphA2 mutants, respectively), the strains impaired only in cyanophycinase gene cphB1 or in cphB1 and cphB2 showed more pronounced defects in diazotrophic growth, growth rates being 64 and 62% of the wild-type value for the single mutant cphB1 and the double mutant cphB1 cphB2, respectively. Strain CSS21, impaired only in cphB2, showed no noticeable impairment in diazotrophic growth.

Filaments from diazotrophic cultures of cph mutant strains were microscopically observed and photographed (Fig. 8). Mutants of the cph2 gene cluster, namely strains CSS21 (cphB2), CSS25 (cphA2), and CSS36 (cphB2 cphA2), looked quite similar to the wild-type strain PCC 7120 showing cyanophycin granules at the poles of the heterocysts. In contrast, strains CSS7 (cphA1) and CSS35 (cphB1 cphA1) showed no apparent polar granules in the heterocysts, whereas strain CSS13 (cphB1) showed extensive granulation that could correspond to cyanophycin granules both in vegetative cells and heterocysts. Interestingly, in contrast to the wild type, in strain CSS13 granules were not located at the heterocyst poles. The double mutant of the cyanophycin synthetase genes (strain CSS27) looked much like that of cphA1 (strain CSS7), and the double mutant of the cyanophycinase genes (strain CSS23) looked much like that of cphB1 (strain CSS13).

DISCUSSION

The Anabaena sp. PCC 7120 genome contains two clusters of cyanophycin metabolism-like genes that could be identified by similarity to the corresponding sequences of other cyanobacteria. Genes in cluster 1 show higher similarity to previously identified cyanophycinase and cyanophycin synthetase genes.
Anabaena Cyanophycin Metabolism Genes

Cyanophycin content and diazotrophic growth rate of Anabaena sp. PCC 7120 and mutants impaired in cph genes

| Strain | Genotype | Cyanophycin content* | Growth rate constant μ |
|--------|----------|----------------------|------------------------|
| PCC 7120 | Wild type | 636 ± 98 (n = 3) | 0.72 ± 0.08 (n = 5) |
| CSS7 | cphA1::C.S3 | 73 ± 8 (n = 3) | 0.65 ± 0.09 (n = 5) |
| CSS13 | cphB1::C.K3 | 1517 ± 157 (n = 3) | 0.46 ± 0.12 (n = 5) |
| CSS35 | cphA1::C.S3, cphB1::C.K3 | 89 ± 32 (n = 2) | ND |
| CSS25 | cphA2::EmR | 499 ± 41 (n = 2) | 0.68 ± 0.04 (n = 5) |
| CSS21 | cphB2::C.S3 | 825 ± 109 (n = 3) | 0.74 ± 0.11 (n = 5) |
| CSS36 | cphA2::EmR, cphB2::C.S3 | 562 ± 74 (n = 2) | ND |
| CSS27 | cphA1::C.S3, cphA2::EmR | 69 ± 11 (n = 3) | 0.62 ± 0.09 (n = 3) |
| CSS23 | cphB1::C.K3, cphB2::C.S3 | 1505 ± 250 (n = 3) | 0.45 ± 0.06 (n = 4) |

* Cyanophycin content of Synechococcus sp. PCC 7942 was analyzed as a negative control of the assay, rendering a value of 68 μg of Arg-mg of Chl−1.
ND, not determined.

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than those of cluster 2. The study of the phenotype of mutant strains derived from Anabaena sp. PCC 7120 bearing inactivated versions of cph genes (Table I, Fig. 8) showed that, at least under the conditions used in this work, gene cphA1 is responsible for most of the cyanophycin synthetase activity, and cphB1 is responsible for the bulk of cyanophycin degradation. This appears to be the case in vegetative cells and heterocysts. Nonetheless, cphA2 and cphB2 genes also make some contribution to cyanophycin accumulation and degradation, respectively.

Both cphB1 and cphA1 genes are expressed in whole filaments growing with ammonium, nitrate, or N₂ as nitrogen sources, but expression is higher in the combined nitrogen-free medium. In diazotrophic cultures, once N₂-fixing heterocysts have developed, cphA1 and cphB1 are expressed in these differentiated cells and, taking into account the magnitude of the hybridization signals (Fig. 2) and the heterocyst frequency that is about one-tenth of the total cells of the filament, probably also in vegetative cells. It also appears that in heterocysts, the cphA1 gene is expressed more strongly than cphB1 (Fig. 2, a and b). Whereas cphB1 shows hybridization to heterocyst transcripts of up to ~5 kb, similar to those hybridizing to cphB1 and cphA1 in whole filaments, cphA1 shows hybridization preferably to heterocyst transcripts of smaller sizes, ~3 kb, similar to those found to hybridize with the cphA1 probe in whole diazotrophic filaments of cphB1 mutant strain CSS13 (Fig. 2c). Collectively, these observations suggest that cphB1 and cphA1 can be cotranscribed in vegetative cells and heterocysts, although in these differentiated cells cphA1 is expressed preferably as a monocistronic message resulting in a higher expression than that of cphB1. This interpretation is consistent with the finding of mRNA 5' ends localized both upstream of cphB1 and in the intergenic region between cphB1 and cphA1 (Fig. 3).

Upstream of cphB1 three different 5' transcript ends were found. RNA molecules with 5' ends Iβ and IIβ would be generated from nitrogen-regulated promoters that are induced upon transfer from ammonium-containing cultures to media lacking combined nitrogen. Whereas full activation of promoter P_{cphB1-3} appears to take place early upon the transference, activation of P_{cphB1-1} seems maximal only at later times, when fully developed heterocysts are present in the culture (Figs. 3a and 4c). Thus, whereas P_{cphB1-3} may be used in vegetative cells of filaments incubated in the absence of combined nitrogen, P_{cphB1-1} seems to be the main cphB1 promoter used in heterocysts (Fig. 3a). Activation of both P_{cphB1-1} and P_{cphB1-3} is dependent on the global nitrogen control transcriptional regulator NtcA (Fig. 4c), and indeed specific NtcA binding to DNA fragments encompassing the regions upstream of the tsp of each of these promoters is observed in vitro (Fig. 5). Both of these tps are preceded by sequences conforming to the −10 box of NtcA-activated promoters (see Ref. 4), although none of them shows a canonical NtcA-binding site in the most frequent position (−22 nucleotides upstream of the −10 box) (Fig. 9a). The observed binding of NtcA to DNA fragment B (Fig. 5c) probably takes place at the canonical NtcA-binding sequence GTATCTAAAAGTAC centered at position −92.5 from tsp1β (Fig. 9a). This is a position fully compatible with a transcription activation mechanism similar to that of the homologous regulator CAP at Class I activated promoters (39). On the other hand, because no canonical NtcA binding box can be found in fragment C, binding of NtcA at promoter P_{cphB1-1} (Fig. 5d) should take place at a sequence imperfect with regard to the consensus of NtcA binding sites. Indeed, NtcA has been shown to bind to some Anabaena promoters with putative NtcA binding sites that resemble but do not match the consensual NtcA-binding box (4).

Because the NtcA binding site of promoter P_{cphB1-1} is downstream from tsp IIIβ (Fig. 9a), it is possible that binding of NtcA to this site has a negative effect on transcription initiated at tsp IIβ. On the other hand, affinity of NtcA for the P_{cphB1-3} promoter appears to be higher than for P_{cphB1-1}. NtcA-regulated transcription of the cphB4 operon would thus consist of activation of P_{cphB1-3} upon combined nitrogen deprivation, long
before heterocysts have completed differentiation, consistent with the presence of 5’ transcript end III\* at early times after combined nitrogen deprivation, followed by activation of \( P_{cphB1-1} \) in heterocysts, consistent with the presence of 5’ transcript ends II\* at later times after combined nitrogen deprivation (Fig. 4c). Binding of NtcA to \( P_{cphB1-1} \) would be possible given the high levels of active NtcA protein probably present in heterocysts, and it could in turn inhibit transcription from \( P_{cphB1-3} \) in heterocysts, consistent with the fact that 5’ transcript end III\* was the most abundant in these cells. Additionally, the putative tsp corresponding to 5’ RNA end II\* is preceded by sequences matching the −10 box and resembling the −35 box of \( \sigma^{70} \) consensus-like promoters (Fig. 9a), which would be responsible for its constitutive expression in whole filaments. Given the low relative abundance of this transcript end in RNA preparations from heterocysts, it could be speculated that operation of \( P_{cphB1-2} \) is also inhibited by the operation of \( P_{cphB1-1} \).

In the intergenic region between \( cphB1 \) and \( cphA1 \), three 5’ transcript ends were identified. The 5’ ends IA and IIIA are found with similar abundance in whole filaments under the different nitrogen conditions tested (Fig. 3b). Whereas the putative tsp IIIA is preceded by sequences matching the \( \sigma^{34} \) box and resembling the \( \sigma^{34} \) consensus promoters, only sequences matching the \( \sigma^{70} \) box could be recognized upstream of IA, although in this case sequences that could represent an UP element are also found (Fig. 9b). Thus, 5’ ends IA and IIIA seem to represent true constitutive promoters, although the similar pattern of occurrence of these ends makes it conceivable that IA might originate from the same transcript producing IIIA. The abundance of 5’ transcript II\* is higher in established diazotrophic cultures than in those using combined nitrogen deprivation.
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and is the most abundant in heterocysts (Fig. 3b). Specific NtcA binding to the cphB1-cphA1 intergenic DNA region has been observed, NtcA showing higher affinity for this region than for any of the sites upstream of cphB1 (Fig. 6). In this intergenic region, NtcA binding can take place at a sequence, GTACA\GGGTTAG, centered at nucleotide −49.5 from tsp IIb (Fig. 9b) that resembles the canonical NtcA binding sequence. This putative NtcA-binding site is separated by 21 bp from a putative −10 box (Fig. 9b), thus conforming to the canonical structure of the more common (Class II) NtcA-activated promoters. These results suggest that the promoter corresponding to IIa (P\textsubscript{cphA1-2}), which represents the main promoter for monocistronic expression of cphA1 in heterocysts (Fig. 3b), is activated by NtcA. The location of the P\textsubscript{cphA1-2} promoter NtcA-binding box overlapping the −10 box of P\textsubscript{cphA1-3} (Fig. 9b) could lead to a negative effect of NtcA binding on transcription from P\textsubscript{cphA1-3}, consistent with the observation that the abundance of 5′ transcript end III\textsubscript{R} is low in heterocysts (Fig. 3b). In addition, tsp II\textsubscript{b} is preceded by sequences that resemble the −35 box of σ\textsuperscript{70} consensus promoters (Fig. 9b) that, together with the −10 box that would be shared by the NtcA-activated promoter, could also promote transcription starting at the same tsp. This −35 box would be used preferably in vegetative cells and would thus be responsible for the presence of 5′ end II\textsubscript{R} in whole filaments grown under any nitrogen condition as well as for its apparent independence of the ntcA mutation (Fig. 4d).

In summary, besides cotranscription of cphB1 and cphA1 genes, monocistronic transcription of cphA1 can take place in Anabaena sp. PCC 7120 both in vegetative cells, from the consensus P\textsubscript{cphA1-3} promoter (and perhaps also from P\textsubscript{cphA1-1}) and from the bivalent P\textsubscript{cphA1-2} promoter, and in heterocysts from an NtcA-dependent operation of P\textsubscript{cphA1-2}. The preferential operation in heterocysts of NtcA-activated promoters P\textsubscript{cphB1-1} and P\textsubscript{cphA1-2} could respond for the higher extractable activities of both cyanophycin synthetase and cyanophycinase that have been found in heterocysts with respect to vegetative cells (e.g. see Ref. 11). Based on the observation that cphB and cphA from Synechocystis sp. PCC 6803 can independently be expressed in E. coli when cloned in the two possible orientations, it has been suggested that the cphB-cphA gene cluster would not be co-transcribed in this cyanobacterium (12). Additionally, cyanophycin synthetase and cyanophycinase of different cyanobacteria can show different relative abundances under different physiological conditions. We have shown here that, in Anabaena sp. PCC 7120, these observations can be accounted for by differential regulation of different promoters directing the synthesis of cphB1-cphA1 polymeric or cphA1 monocistronic messages under different physiological conditions and in different cell types in the diazotrophic filament. Nonetheless, it is worth stressing that the whole system appears to be set to ensure a good level of expression of both genes under all conditions tested.

A second cluster of cyanophycin-related genes has been identified in Anabaena sp. PCC 7120. In contrast to the situation with cluster cphA1, genes in cluster cph2 are found in opposite orientations (Fig. 1) and are expressed as monocistronic messages in whole filaments grown with ammonium, nitrate, or N\textsubscript{2}, although expression seems also to be higher in the absence of ammonium (Fig. 7). However, expression of cph2 genes was lower than that of cphA1 genes precluding their detailed analysis. Low expression of cph2 genes is also consistent with the low metabolic impact of cph2 gene products in strain PCC 7120. Organization of cph genes in bacteria studied to date conforms to three different structures: (i) the cphB-cphA disposition found in cyanobacteria, including the cph1 cluster of Anabaena sp. PCC 7120, and also found in Clostridium botulinum strain ATCC 3802 and D. hafniense strain DCB-2; (ii) a cphA′-cphA disposition found in Bordetella bronchiseptica strain RB-50, Bordetella pertussis strain Tohama I, Bordetella parapertussis strain 12822, and Nitrosomonas europaea strain ATCC 25578; and (iii) a cphA-cphH (in which cphH would encode a kind of fusion protein of two cyanobacterial-like CphH units) found in Acinetobacter sp. strain ADP1 (14). The organization of the Anabaena sp. PCC 7120 cph2 gene cluster represents a fourth model of cyanophycin metabolism gene organization in bacteria.

The ability to grow under diazotrophic conditions was also studied in the cph mutant strains of Anabaena sp. PCC 7120. In heterocysts, conspicuous accumulation of cyanophycin takes place at the poles that are adjacent to vegetative cells. Mutant strains impaired in cyanophycin synthetase genes (cphA1 and/or cphA2) exhibit growth rates on N\textsubscript{2} only slightly lower than that of the wild-type strain. Consistent with these results are those of Ziegler et al. (40), showing that in a cyanophycin synthetase mutant of Azotobacter variabilis ATCC 29413 growth on N\textsubscript{2} was somewhat reduced only under high, nonlimiting light conditions. In contrast, diazotrophic growth rates of mutants impaired in cyanophycinase activity (cphB1 and cphB1 cphB2 strains) are significantly lower than that of the wild type (Table 1). Microscopic observation (Fig. 8) shows that cphA1 mutant strains exhibit no apparent polar granules in the heterocysts, whereas cphB1 mutants show extensive granulation both in the vegetative cells and in heterocysts. In these strains, heterocysts look clearly different from those of the wild type. Cyanophycin accumulated as polar granules in heterocysts could be formed at the expense of amino acids synthesized after N\textsubscript{2} fixation, and it can constitute a dynamic reservoir of fixed nitrogen (e.g. see Ref. 8), although our results suggest that the path through cyanophycin of newly synthesized organic nitrogen in the heterocyst is a dispensable, rather than an obligatory, route (little effect of cyanophycin synthetase inactivation).

In contrast, the accumulation of amino acids into cyanophycin that could not be easily degraded (in mutants exhibiting low cyanophycinase activity) probably represents a sink of fixed nitrogen that is detrimental for diazotrophic growth.

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