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The *hetC* Gene Is a Direct Target of the NtcA Transcriptional Regulator in Cyanobacterial Heterocyst Development

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The heterocyst is the site of nitrogen fixation in aerobically grown cultures of some filamentous cyanobacteria. Heterocyst development in *Anabaena* sp. strain PCC 7120 is dependent on the global nitrogen regulator NtcA and requires, among others, the products of the *hetR* and *hetC* genes. Expression of *hetC*, tested by RNA-DNA hybridization, was impaired in an *ntcA* mutant. A nitrogen-regulated, NtcA-dependent putative transcription start point was localized at nucleotide −571 with respect to the *hetC* translational start. Sequences upstream from this transcription start point exhibit the structure of the canonical cyanobacterial promoter activated by NtcA, and purified NtcA protein specifically bound to a DNA fragment containing this promoter. Activation of expression of *hetC* during heterocyst development appears thus to be directly operated by NtcA. NtcA-mediated activation of *hetR* expression was not impaired in a *hetC* mutant, indicating that HetC is not an NtcA-dependent element required for *hetR* induction.

Cyanobacteria are phototrophic bacteria that carry out oxygenic photosynthesis and likely represent the phylogenic ancestors of the chloroplasts of eukaryotic algae and higher plants. Cyanobacteria obtain cellular nitrogen mainly from inorganic sources such as nitrate or ammonium, and many can also use atmospheric nitrogen as a nitrogen source (9, 10). The assimilation of nitrogen by cyanobacteria is subjected to tight regulation so that ammonium is assimilated with preference over other good nitrogen sources such as urea, nitrate, nitrite, or N_2 when more than one are available (10). At the molecular level, this possibility of choice is based on a nutritional repression exerted by ammonium on the expression of genes involved in the assimilation of alternative nitrogen sources, and *ntcA* has been identified as a gene that encodes a transcriptional regulator exerting global nitrogen control that appears to be universally distributed in cyanobacteria (11, 13). The NtcA protein belongs to the cyclic AMP receptor protein family of bacterial regulators and bears close to its C-terminal end a helix-turn-helix motif for interaction with DNA (31). NtcA binds to specific sites in the promoter regions of regulated genes involved in nitrogen assimilation, activating their expression in response to ammonium withdrawal (22). The structure of the cyanobacterial NtcA-activated promoter comprises a −10 box in the form TAN_3T and an NtcA-binding site containing the sequence signature $GTAN_8TAC$ that is located 20 to 23 nucleotides upstream from the −10 box and that appears to substitute for the −35 box that would be present in promoters similar to the canonical *Escherichia coli* σ^{70} promoters (11, 22).

Some filamentous cyanobacteria are able to differentiate, under conditions of aerobiosis and combined nitrogen deprivation, cells specialized in nitrogen fixation called heterocysts, which differentiate from vegetative cells located at semiregular intervals in the filament (5, 35). Heterocysts differ from vegetative cells in many structural and functional features that turn them into efficient factories for nitrogen fixation. At the molecular level, the differential physiology of the heterocyst is supported by a pattern of gene expression that largely differs from that taking place in vegetative cells (5, 35).

In recent years, a number of genes of *Anabaena* sp. strain PCC 7120 whose function is required for heterocyst development have been identified (5, 17, 34, 35). The products of some of them (such as *devA* [23], *hetM* [7], and *hepA* [20]) serve a structural role, and their inactivation leads to the differentiation of aberrant heterocysts, in most cases carrying an imperfect heterocyst envelope. For others, e.g., *hetR* (3, 4) and *hetC* (21), the actual function in heterocyst development is still unknown although their inactivation leads to a lack of differentiation. Also, the *patS* gene, whose inactivation leads to the differentiation of supernumerary heterocysts, has been described (36). HetR has recently been suggested to have protease activity (37). *hetC* would encode a 1,044-amino-acid protein belonging to the superfamily of the ATP-binding cassette (ABC) transporters, with the greatest similarity to proteins of the HlyB family that facilitate the export of toxic proteins. Since the mutation of *hetC* prevents heterocyst differentiation, it has been reasoned that HetC could be involved in the export of an inhibitor of differentiation (21). The expression of *hetR* (3, 4) and *hetC* (21) increases in response to combined nitrogen deprivation. The expression of *devA*, *hetM*, and *hepA* (7), as well as of the *nifHDK* operon encoding nitrogenase (8, 16), is also activated upon combined nitrogen deprivation in a HetR-dependent but, to date, unknown manner.

The *ntcA* gene has been shown to be necessary for N_2 fixation and heterocyst development in *Anabaena* sp. strain PCC 7120 (12, 33). Mutant strains bearing an inactivated version of *ntcA* are unable to grow on N_2, as well as on nitrate, and do not show any sign of heterocyst differentiation upon combined nitrogen deprivation. Moreover, these *ntcA* mutants do not show the activation of *hetR* or of *nifHDK* expression that takes place in the wild-type strain in response to nitrogen stepdown (12).

Elucidation of the direct targets of NtcA during heterocyst differentiation is crucial for understanding the mechanism by which gene expression is regulated for the development and function of the cyanobacterial heterocyst. In this report, we

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present a study of the expression of the *hetC* gene and provide evidence for a direct role for NtcA as an activator of the expression of this gene.

MATERIALS AND METHODS

Bacterial strains. This study was carried out with the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 and two Het$^-$ derivatives, strain CSE2 (an insertional mutant of the *ntcA* gene) (12) and strain DR1653 (an insertional mutant of the *hetC* gene) (21). They were grown photoautotrophically at 30°C in $BG11_0C$ medium ($BG11_0$ medium [26] supplemented with 0.84 g of $NaHCO_3$ per liter), bubbled with a mixture of CO_2 (1% [vol/vol]) and air, and supplemented with 2 μg of streptomycin and 2 μg of spectinomycin · ml^{-1} for strains CSE2 and DR1653. When indicated, 8 mM NH_4Cl (plus 16 mM TES [*N*-tris{hydroxymethyl}methyl-2-aminoethanesulfonic acid]–NaOH buffer [pH 7.5]) or 17.6 mM $NaNO_3$ was added as a nitrogen source.

For RNA isolation, cells growing exponentially in $BG11_0C$ medium supplemented with NH_4Cl were harvested at room temperature and either used directly or washed with $BG11_0C$ medium, resuspended in $BG11_0C$ medium (nitrogen free) supplemented or not with NH_4Cl or $NaNO_3$, and further incubated under culture conditions for the number of hours indicated in the figure legends.

E. coli DH5α (Bethesda Research Laboratories) was used for all plasmid constructions, except for pCSAM70 (see below). *E. coli* BL21(DE3) (28) was used in this case. Both *E. coli* strains were grown in Luria broth (LB) as described previously (27). *E. coli* strains containing pCSAM70 were grown in LB medium supplemented with 0.2% glucose in order to reduce basal expression of the *ntcA* gene.

Plasmids. Plasmid pCSAM70 contains a ca. 1.6-kb DNA fragment from the *ntcA* region of *Anabaena* sp. strain PCC 7120 cloned into *Bam*HI- and *Hin*dIII-digested, Klenow fragment-filled expression vector pQE9 (Qiagen). This fragment extends from the start codon of the *ntcA* gene (cloned in frame with the site of initiation of translation located after the IPTG [isopropyl-β-D-thiogalactopyranoside]-inducible promoter in pQE9) to the first *Hin*cII site located downstream of *ntcA* (13). The NtcA protein encoded in pCSAM70 contains an N-terminal histidine tag (see below). pCSAM70 was maintained in *E. coli* cells bearing plasmid pREP4 (Qiagen), which express high levels of the LacI^q repressor.

Plasmid pCSAM83 contains an 853-bp fragment from the upstream region, and the initial part of the coding sequence, of the *hetC* gene from *Anabaena* sp. strain PCC 7120 (GenBank accession no. U55386) (21). This fragment was amplified by PCR with oligonucleotides HC1 (5′-TAGTACATCGGTGAGGG GTG-3′; corresponding to positions −693 to −674 relative to the translation start of *hetC*) and HC4 (5′-GCCGAACTACCCAGTTTTGG-3′; complementary to positions +160 to +141 relative to the translation start of *hetC*) and chromosomal DNA from strain PCC 7120 as the template and cloned into plasmid pGEM-T (Promega).

Plasmid pCSAM86 contains a 1,598-bp fragment internal to the *hetC* gene from *Anabaena* sp. strain PCC 7120. This fragment was amplified by PCR with oligonucleotides HC5 (5′-AGAGTTGAGCCAAAACTGG-3′; corresponding to positions +132 to +150 relative to the translation start of *hetC*) and HC6 (5′-GTAAGGGTAACTGCAACG-3′; complementary to positions +1729 to +1712 relative to the translation start of *hetC*) and chromosomal DNA from strain PCC 7120 as the template and cloned into plasmid pGEM-T (Promega).

DNA and RNA isolation and manipulation. Total DNA (6) and RNA (14; based on reference 18) from *Anabaena* sp. strain PCC 7120 and its derivatives were isolated as previously described. Sequencing was carried out by the dideoxy chain termination method with a T^{7}Sequencing kit (Pharmacia Biotech) and α-^{35}S-thio dATP. DNA fragments were purified from agarose gels with the Geneclean II kit (Bio 101, Inc.).

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 (1, 27).

Northern blotting and hybridization. For Northern analysis, 70 μg of RNA was loaded per lane and electrophoresed in 1% agarose denaturing formaldehyde gels. Transfer and fixation to Hybond-N$^+$ membranes (Amersham Pharmacia) were carried out with 0.1 M NaOH. Hybridization was performed at 65°C according to the recommendations of the manufacturer of the membranes. The *hetC* probe was amplified by PCR with oligonucleotides HC5 and HC6 (see above) and pCSAM86 as the template. The *hetR* probe was a 703-bp *Hae*II fragment containing most of the *hetR* gene (4). Fragments used as probes were labeled with a Ready to Go DNA labeling kit (Pharmacia Biotech) by using [α-^{32}P]dCTP. Images of radioactive filters were obtained and quantified with a Cyclone storage phosphor system and OptiQuant image analysis software (Packard).

Primer extension analysis. Oligonucleotides used for primer extension analysis of the *hetC* transcript were HC2 (5′-TGTGAGCAACATCGACATCTG-3′; complementary to positions −411 to −431 relative to the translation start of *hetC*), HC3 (5′-CGGCATTTTAATGTACTGCC-3′; complementary to positions −85 to −104 relative to the translation start of *hetC*), HC4 (see above), and HC7 (5′-GGAAAAGGTTCTCTATGAAC-3′; complementary to positions −348 to −367 relative to the translational start of *hetC*). Plasmid pCSAM83, which contains the upstream region of the *hetC* gene, was used to generate dideoxy-sequencing ladders with the same primers.

Oligonucleotides were end-labeled with T4 polynucleotide kinase (Boehringer) and [γ-^{32}P]dATP as described previously (1) and mixed with 25 μg of total RNA in the presence of 10 mM Tris-HCl (pH 8.0)–150 mM KCl–1 mM EDTA. The mixtures were incubated first at 85°C for 10 min for denaturation of RNA and then at 50°C for 1 h for annealing. The extension reactions were carried out at 47°C for 1 h in a final volume of 45 μl containing the whole annealing reaction mixture, 0.25 mM (each) deoxynucleoside triphosphate, 200 U of reverse transcriptase (Superscript II; Gibco-BRL), and the buffer recommended by the transcriptase provider. Reaction mixtures were then treated with RNase A (DNase free; Boehringer) and extracted with phenol. The extended fragments were precipitated with sodium acetate and ethanol, resuspended in formamide loading dye, and loaded onto 6% polyacrylamide-urea sequencing gels next to the corresponding sequencing ladder. Images of radioactive gels were obtained and quantified as described above.

Overproduction and purification of histidine-tagged NtcA. For purification of histidine-tagged NtcA, saturated cultures of *E. coli* BL21(DE3) (pREP4, pCSAM70) grown in the presence of 0.2% glucose were centrifuged, diluted 1:50 in LB medium without glucose, and incubated for an additional 2-h period under culture conditions. IPTG was added at 1 mM, and the incubation was continued for three more hours. Cells from 500 ml of cultures were collected, washed with 1 volume of 20 mM sodium phosphate buffer (pH 7.0) containing 200 mM NaCl and 10% glycerol, and resuspended in 5 ml of the same buffer per gram of cells. After the addition of 1 mM phenylmethylsulfonyl fluoride, the cells were disrupted by sonication and the extract was centrifuged at 10,000 × *g* for 15 min. The resulting crude extract was chromatographed through a 1-ml chelating Sepharose Hitrap column (Pharmacia Biotech) charged with $CuSO_4$, by using a fast protein liquid chromatography system (Pharmacia Biotech). Contaminating proteins were eluted by washing the column with 15 volumes of the same buffer. Bound NtcA was eluted with a linear gradient of imidazole (0 to 0.5 M) in the same buffer as that described above. Pure NtcA, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), started to elute from the column at about 200 mM imidazole.

Proteins in whole cells or crude extracts were analyzed by standard electrophoresis in SDS-PAGE gels followed by staining with Coomassie brilliant blue R. Protein concentration was estimated by a dye-binding assay (Bio-Rad).

Band-shift assays. DNA fragments to be used in electrophoretic mobility shift assays were obtained by PCR amplification. Oligonucleotides HC1 and HC2 (see above) and plasmid pCSAM83 were used for the *hetC* upstream region. For the *glnA* upstream region, oligonucleotides GA3 (5′-GGATTTTATGTCAAAGTT GACCCC-3′; corresponding to positions −238 to −215 relative to the translation start of *glnA*) and GA6 (5′-CGAAACAAAGTTGATGAC-3′; complementary to positions −70 to −87 relative to the translation start of *glnA*) and plasmid pAN503 (29) were used. The same unlabeled DNA fragments were added as competitors in some assays. Alternatively, an unrelated, unlabeled DNA fragment from plasmid pBluescript obtained by PCR amplification with M13 reverse and forward sequencing primers was used. DNA fragments were end-labeled with T4 polynucleotide kinase (Boehringer) and [γ-^{32}P]dATP as described previously (1). Assays were carried out as described previously (22) with 0.05 pmol of labeled fragment and 5 pmol of purified histidine-tagged NtcA. Assays carried out with *E. coli* crude extracts contained 0.85 μg of total protein carrying, for the extract from IPTG-induced *E. coli* BL21(DE3) (pREP4, pCSAM70) cells, approximately 1.5 pmol of histidine-tagged NtcA. Unlabeled competitor fragments were added in a 25-fold molar excess. Images of radioactive gels were obtained with a Cyclone storage phosphor system (Packard).

RESULTS

Nitrogen-regulated transcription initiation of the *hetC* gene. To study transcriptional regulation of *hetC*, primer extension experiments were carried out with oligonucleotides HC2, HC3, HC4, and HC7 by using RNA isolated from cells grown on ammonium and incubated for 6 h in medium containing no combined nitrogen, nitrate, or ammonium. With oligonucleotide HC2, a major RNA 5′ end that would correspond to a transcription start point (tsp) situated at position −571 with respect to the translational start of the *hetC* gene was detected (Fig. 1A). The use of this putative tsp was dependent on the nitrogen regime of the cells, being most efficient in the absence of combined nitrogen and more efficient in nitrate- than in ammonium-containing medium. With oligonucleotide HC7 (not shown), the putative tsp located at −571 was confirmed, whereas the bands showing up at position −474/−477 in Fig. 1 were not detected. No other tsp was detected with oligonucleotide HC3 or HC4.

To determine the time course of activation of this *hetC* tsp

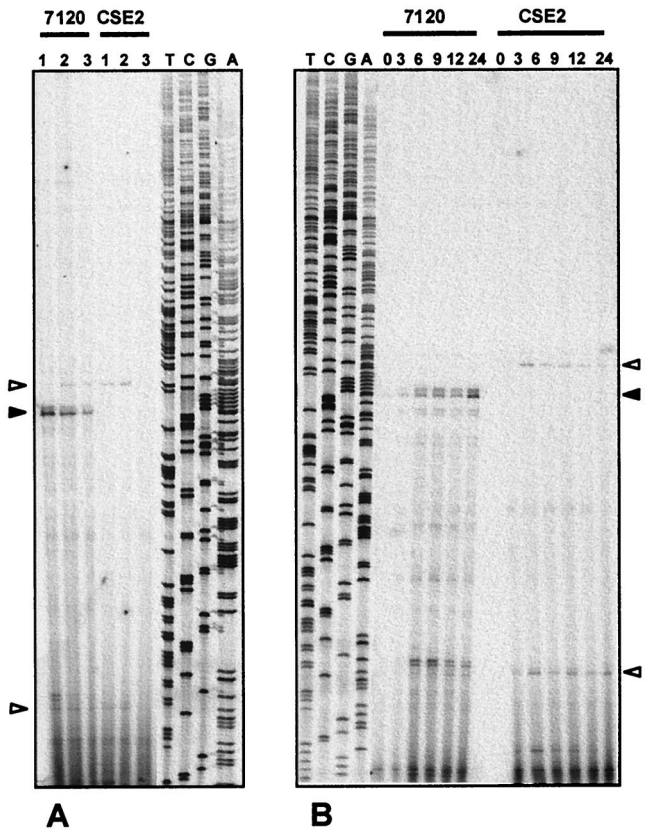

FIG. 1. Primer extension analysis of expression of the *hetC* gene in *Anabaena* sp. strain PCC 7120 and mutant strain CSE2 (mutant *ntcA*). (A) Primer extension assays were carried out with RNA isolated from cells grown on ammonium and incubated for 6 h in medium lacking combined nitrogen (lanes 1) or containing nitrate (lanes 2) or ammonium (lanes 3). (B) Time course of expression of the *hetC* gene in *Anabaena* sp. strain PCC 7120 and mutant strain CSE2 upon combined-nitrogen stepdown. Primer extension assays were carried out with RNA isolated from cultures grown on ammonium (lanes 0) or grown on ammonium and incubated in combined-nitrogen-free medium for 3, 6, 9, 12, or 24 h. Assays were carried out with oligonucleotide HC2 (see Materials and Methods). The sequencing ladders shown were generated with the same oligonucleotide and plasmid pCSAM83. Solid arrowheads point to the putative tsp identified at position −571. Open arrowheads point to the −474/−477 (bottom) and −581 (top) positions (see text).

upon combined-nitrogen deprivation, *Anabaena* sp. strain PCC 7120 cells grown with ammonium were transferred to medium lacking combined nitrogen, and primer extension assays were carried out with RNA extracted from the cultures at several time points after ammonium withdrawal. As shown in Fig. 1B, the abundance of the RNA that would be synthesized from the tsp located at position −571 increased more conspicuously during the first 6 h and then continued to increase up to at least 24 h after the transfer to the combined-nitrogen-free medium, a time at which, under our culture conditions, fully differentiated heterocysts were already present in the cultures.

Regulation by NtcA of *hetC* expression. The pattern of expression of *hetC* in response to the nitrogen regime of the cells is consistent with that expected for an NtcA-regulated gene. To test the involvement of the transcriptional regulator NtcA in the control of *hetC* expression, Northern and primer extension assays were performed with RNA isolated from cells of mutant strain CSE2, which carries an insertionally inactivated *ntcA* gene (12), subjected to combined-nitrogen stepdown, and the results obtained were compared to those obtained with RNA from the wild-type strain PCC 7120. No RNA hybridizing to the *hetC* probe could be detected in strain CSE2 (Fig. 2). Repeated attempts to isolate intact transcripts from the *hetC* gene were unsuccessful; thus the observed signal in Northern blots corresponds to degradation products of the *hetC* transcript which, according to the location of the putative tsp and the size of the predicted *hetC* product (1,044 amino acids), should be at least 3.6 kb long. Additionally, no expression of *hetC* from the nitrogen-regulated tsp located at position −571 was detected in this mutant (Fig. 1). (The faint band at position −581 that shows up in strain CSE2 [Fig. 1] could be attributable to a putative weak σ^{70}-type promoter whose −35 box, in the form GTAACA, would overlap the NtcA-binding site [see below].)

Binding of purified NtcA to the promoter region of the *hetC* gene. Thirty-three nucleotides upstream from the nitrogen-regulated tsp of *hetC* determined above, sequence GTAACA TGAGATAC is found (21); this sequence conforms to the consensus sequence for NtcA-binding sites on DNA (11, 22). It is located about 605 bp upstream from the putative translation start of the *hetC* gene. In order to test binding of NtcA to the promoter region of *hetC*, histidine-tagged NtcA from *E. coli* cells bearing plasmid pCSAM70 was overproduced and purified. The NtcA protein encoded in pCSAM70 contains a 12-amino-acid N-terminal extension (MetArgGlySer $[His]_6$GlySer-) preceding the complete native *Anabaena* sp. strain PCC 7120 NtcA sequence (with the only exception that Ile-2 is replaced by Val) and can thus be purified by immobilized metal ion affinity chromatography (see Materials and Methods for details). Figure 3A shows electrophoretic profiles of whole cells of the *E. coli* strains used for overproduction of NtcA, crude extracts from cells treated with IPTG, and a purified NtcA preparation.

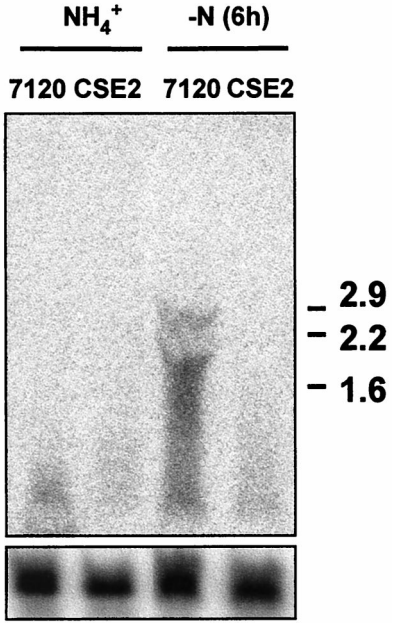

FIG. 2. Northern blot analysis of expression of the *hetC* gene in *Anabaena* sp. strain PCC 7120 and mutant strain CSE2 (mutant *ntcA*). RNA was isolated from ammonium-grown cells (NH_4^+) or from ammonium-grown cells incubated for 6 h in combined-nitrogen-free medium [−N (6 h)]. Hybridization to a probe of the *hetC* gene (upper panel) was carried out as described in Materials and Methods. Samples contained 70 μg of RNA. Hybridization to *rpnB* (32) served as a loading and transfer control (lower panel). Size standards in kilobases are indicated on the right.

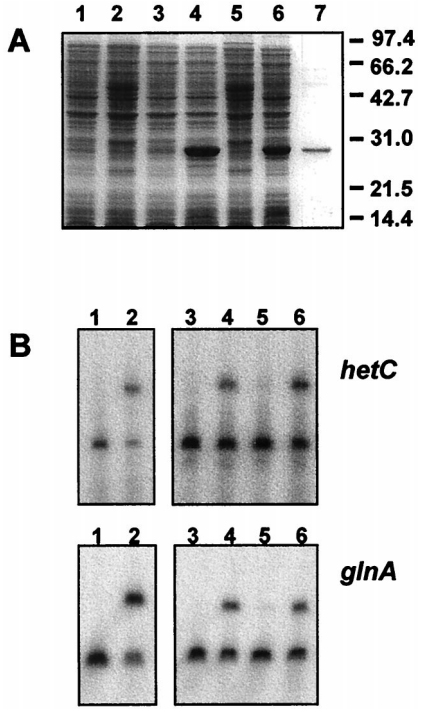

FIG. 3. Overproduction and purification of histidine-tagged NtcA and band-shift assays of a DNA fragment from the *hetC* promoter with purified histidine-tagged NtcA. (A) SDS-PAGE of samples of cultures of *E. coli* BL21(DE3) containing plasmid pREP4 and either vector pQE9 (lanes 1 and 2; 70 μl of culture) or NtcA expression plasmid pCSAM70 (lanes 3 and 4; 100 μl of culture), noninduced (lanes 1 and 3) or induced with IPTG (lanes 2 and 4). Lanes 5 and 6, crude extracts (100 μg of protein) from cultures shown in lanes 2 and 4, respectively; lane 7, 2.4 μg of purified histidine-tagged NtcA. Size standards in kilodaltons are indicated on the right. (B) Band-shift assays with histidine-tagged NtcA. Assays were carried out as described in Materials and Methods with fragments from the upstream regions of *hetC* or *glnA*. Left panels correspond to assays carried out with extracts from *E. coli* BL21(DE3) containing plasmid pREP4 and either vector pQE9 (lanes 1) or NtcA expression plasmid pCSAM70 (lanes 2). Right panels correspond to assays carried out without (lanes 3) or with (lanes 4 to 6) purified histidine-tagged NtcA (5 pmol) without competitor DNA (lanes 4) or with a 25-fold molar excess of the corresponding unlabeled fragment (lanes 5) or an unrelated, unlabeled fragment (lanes 6).

Mobility shift assays were carried out with a 283-bp, ^{32}P-labeled DNA fragment from the promoter region of *hetC* containing the nitrogen-regulated tsp and sequences around it. Electrophoretic retardation of this DNA fragment was effected by the *E. coli* crude extract obtained after induction of the expression of the cloned *ntcA* gene but not by the extract from cells that did not carry a cloned *ntcA* gene (Fig. 3B, upper panel, lanes 1 and 2). Band retardation was also effected by the purified NtcA protein (Fig. 3B, upper panel, lane 4). Retardation of the labeled fragment was effectively competed by the same unlabeled DNA fragment (Fig. 3B, upper panel, lane 5) but not by an unrelated, unlabeled DNA fragment (Fig. 3B, upper panel, lane 6). For the sake of comparison, parallel experiments were carried out with a 171-bp DNA fragment from upstream of the *Anabaena* sp. strain PCC 7120 *glnA* gene, encoding glutamine synthetase, which comprises the P_I promoter and which has previously been shown (12, 24) to bear an NtcA-binding site. Figure 3B, lower panel, shows that this fragment is indeed specifically retarded by NtcA. These experiments indicate a specific binding of NtcA to DNA sequences between positions −692 and −411 relative to the start of the *hetC* gene coding region.

Expression of *hetR* is independent of *hetC*. The expression of *hetR* was investigated by means of RNA-DNA hybridization in strain DR1653, which bears an insertionally inactivated *hetC* gene (21). Induction of *hetR* after nitrogen stepdown took place in the *hetC* mutant in a way similar to that observed in the wild-type strain (Fig. 4), showing that activation of *hetR* expression is independent of *hetC*. The observed *hetR* transcripts, of ca. 1.4 and 1.9 kb, were like those previously reported (4). As a control, expression of *hetR* was also tested with strain CSE2, in which, according to previously reported results (12), no induction of *hetR* was observed.

DISCUSSION

In this work, we have determined a nitrogen-regulated tsp of *hetC* that is located at position −571 with respect to the putative translational start of the gene. This localized the putative NtcA-binding site previously noted upstream from *hetC* (21) in the position characteristic of transcription activator NtcA sites on DNA (Fig. 5). We have additionally shown that purified NtcA specifically binds to a DNA fragment containing the NtcA-binding site in the putative nitrogen-regulated promoter of *hetC*. Moreover, we have found that the *hetC* transcript is barely detectable in an *ntcA* mutant (Fig. 2) and that no transcription from the nitrogen-regulated promoter in that mutant took place (Fig. 1). These results demonstrate a direct transcriptional activation by NtcA of *hetC*. Previous studies of complementation of a *hetC* mutant with *hetC*-containing plasmids had indicated that the region located upstream from position −532 with respect to the *hetC* translational start might be required for stimulation of transcription of *hetC* under nitrogen-deprived conditions (21). This is consistent with the presence of the nitrogen-regulated *hetC* tsp at position −571.

As mentioned above, NtcA is required for activation of the expression of *hetR* (12); HetR is a key regulatory element that acts very early in heterocyst development and that is required for the expression of some other heterocyst development genes (7). However, no DNA sequence similar to the consensus sequence of the NtcA-binding site is present at the *hetR* promoters (cited in reference 19); thus, NtcA may have its induction effect on *hetR* via the activation of another gene(s). We have observed that *hetR* induction after nitrogen stepdown takes place normally in a mutant *hetC* background (Fig. 4), indicating that HetC is not the NtcA-dependent element required for *hetR* induction. Therefore, the NtcA-dependent direct induction of *hetC* described in this work indicates that more than one NtcA-dependent activation event is required for heterocyst development. Even more, NtcA-dependent gene expres-

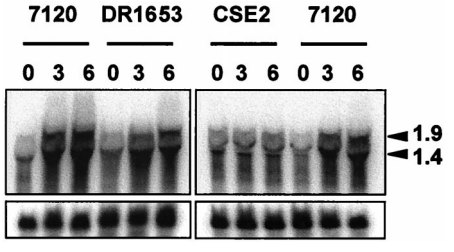

FIG. 4. Northern blot analysis of expression of the *hetR* gene in *Anabaena* sp. strain PCC 7120 and mutant strains DR1653 (mutant *hetC*) and CSE2 (mutant *ntcA*). RNA was isolated from ammonium-grown cells (lanes 0) or from ammonium-grown cells incubated for 3 or 6 h in combined-nitrogen-free medium (lanes 3 and 6, respectively). Hybridization to a probe of the *hetR* gene was carried out as described in Materials and Methods. Samples contained 70 μg of RNA. Hybridization to *rpnB* (32) served as a loading and transfer control (lower panel). Arrowheads, two *hetR* transcripts of 1.4 and 1.9 kb (4).

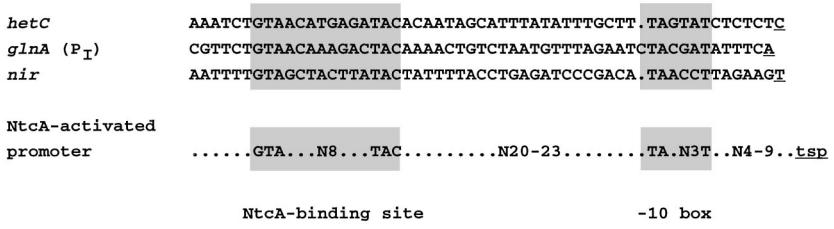

FIG. 5. Nucleotide sequences of the DNA regions upstream of the nitrogen-regulated tsp of three genes of *Anabaena* sp. strain PCC 7120 that have been shown to bear an NtcA-activated promoter. The consensus sequence for NtcA-activated promoters (11, 22) is also shown. The NtcA-binding site and −10 hexamer are indicated by gray boxes. The nucleotide corresponding to the tsp is underlined in each case.

sion appears to take place also in the mature heterocyst. Specific binding of NtcA to the promoter of the *nifHDK* operon has been described previously (24, 30), and we have recently observed that a promoter for the *petH* gene (encoding ferredoxin-NADP$^+$ reductase), used in the heterocysts, is NtcA dependent and binds NtcA in vitro (30). Additionally, the *xisA* gene, which is activated late in the course of heterocyst development (15), seems also to be regulated somehow by NtcA, since the *xisA* upstream sequences bear three NtcA-binding sites (24). All of these data are consistent with the observations that (i) the *ntcA* gene is expressed both during heterocyst development and in mature heterocysts (2, 25) and (ii) heterocyst extracts appear to contain NtcA protein, as detected by means of mobility shift assays (24).

Our data showing a direct activation of the *hetC* promoter by NtcA suggest that expression of *hetC* responds to the environmental cue of nitrogen deficiency and represent the first determination of the mechanism by which regulation of expression of a gene involved in the differentiation of the cyanobacterial heterocyst is operated at the molecular level.

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