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Anabaena sp. Strain PCC 7120 *ntcA* Gene Required for Growth on Nitrate and Heterocyst Development

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The *Anabaena* sp. strain PCC 7120 *ntcA* (*bifA*) gene encodes a sequence-specific DNA-binding protein, NtcA (BifA, VF1) that interacts with the upstream region of several genes, including *glnA*, *xisA*, *rbcL*, and *nifH*. We have constructed a *ntcA* null mutant by interrupting the gene with an Ω Sp^r-Sm^r cassette. The *ntcA* mutant was not able to grow with nitrate or atmospheric dinitrogen as the sole nitrogen source but could be grown on medium containing ammonium. The *ntcA* mutant was unable to form heterocysts and did not rearrange the *nifD* or *fdxN* elements after induction on a medium lacking combined nitrogen. Northern (RNA) analysis of *ntcA* in the wild-type strain during nitrogen stepdown showed a peak of *ntcA* message at an early stage (12 h) of heterocyst induction. Complementation of the *ntcA* mutant with a DNA fragment containing the *ntcA* gene and 251 bp of upstream sequence on a shuttle vector restored a wild-type phenotype; however, a similar construction containing 87 bp of upstream sequence only partially restored the phenotype. Northern analysis of RNA samples isolated from ammonium-grown cultures of the *ntcA* mutant showed reduced amounts of *glnA* message and the absence of a 1.7-kb transcript. In the wild type, the 1.7-kb transcript represents the majority of *glnA* transcripts after nitrogen stepdown. The *ntcA* mutant showed a normal pattern of *rbcLS* messages under these growth conditions.

Anabaena sp. strain PCC 7120 is a diazotrophic filamentous cyanobacterium. In the presence of combined nitrogen, the filaments are composed entirely of photosynthetic vegetative cells. Under nitrogen-limiting conditions, about 10% of the cells along the filament undergo patterned differentiation to form heterocysts. Heterocysts are highly specialized, terminally differentiated cells responsible for nitrogen fixation (41). Heterocysts provide the filament with fixed nitrogen in the form of glutamine and, in return, are provided with the carbohydrate products of photosynthesis.

Vegetative cells and heterocysts show distinct differences in their ultrastructure, biochemical processes, and gene expression (6). In developing heterocysts, a new external cell envelope containing glycolipid and polysaccharide layers is formed to limit oxygen diffusion into heterocysts and protect the oxygen-labile nitrogenase. In addition, the photosynthetic membrane is reorganized. Photosystem II is inactivated, although photosystem I is preserved. Changes in gene expression include the induction of nitrogen fixation (*nif*) genes and the repression of genes required for carbon fixation, such as *rbcLS*.

At least two genome rearrangements occur in heterocysts: excision of an 11-kb element from the *nifD* gene (20) and excision of a 55-kb element from the *fdxN* gene (18–20, 30, 31). Both rearrangements are catalyzed by element-encoded site-specific recombinases: XisA (4, 22, 24) and XisF (8), respectively. As one approach to understanding the developmental regulation of these gene rearrangements, we have identified *cis*-acting elements and *trans*-acting factors associated with the *xisA* gene.

The *trans*-acting factor NtcA (BifA, VF1) was initially identified on the basis of its interaction with the *xisA* upstream region (9). NtcA DNA-binding activity is present in both vegetative cells and heterocysts (33). NtcA also binds to the promoter regions of *glnA*, *rbcL*, and *nifH* (33). Analysis of NtcA binding sites identified the consensus binding motif TGT($N_{9\ or\ 10}$)ACA, which is similar to NifA and Crp binding motifs (3, 28, 29). NtcA binds strongly to a single site in the *glnA* promoter region located between the major transcription start site used in vegetative cells (RNA_{II}) and the major transcription start site used under nitrogen-deficient conditions (RNA_I) (33, 36). In contrast, NtcA binds to three adjacent sites and can form three different complexes with the *xisA* upstream region (9).

The PCC 7120 *ntcA* gene (*bifA*) was cloned by an in vivo transcriptional interference selection method (40). The same gene was independently identified in PCC 7120 by Frías et al. by hybridization with the *Synechococcus* sp. strain PCC 7942 *ntcA* gene (17). The predicted NtcA amino acid sequence is similar to those of the family of prokaryotic regulatory proteins represented by Crp (40).

In this study, we show that an *ntcA* mutant of PCC 7120 requires ammonium for growth and is defective for heterocyst formation. We also provide evidence that NtcA may be required for transcription from the *glnA nif*-like promoter.

MATERIALS AND METHODS

Strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown as previously described (21). *Anabaena* sp. strain PCC 7120 and derived strains were grown essentially as previously described (21) except as noted. Standard BG-11 medium contains $NaNO_3$ (17.6 mM) as the nitrogen source (34) but is here designated BG-11($NaNO_3$) for clarity. BG-11(NH_4NO_3) is BG-11($NaNO_3$) supplemented with 2 mM NH_4NO_3 and 5 mM MOPS (3-[*N*-morpholino]propanesulfonic acid) buffer (pH 8.0). $BG\text{-}11_0$ lacks a source of combined nitrogen. For *Anabaena* cultures, the following antibiotics were used at the indicated final concentrations: neomycin, 25 μg/ml; chloramphenicol, 10 μg/ml; erythromycin, 5 μg/ml; and spectinomycin

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Anabaena sp.		
PCC 7120	Wild type	R. Haselkorn
AMC236	PCC 7120 *ntcA*::Ω Sp^r-Sm^r cassette	This study
AMC272	PCC 7120 harboring pAM1323	This study
AMC273	AMC236 harboring pAM1322	This study
AMC274	AMC236 harboring pAM1323	This study
E. coli DH10B	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lac*ZΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara, leu*)*7697 galU galK* λ^- *rpsL nupG*	Bethesda Research Laboratories
Plasmids		
pAM165 (pDW9)	Modified Ω Sp^r-Sm^r cassette in pUC1819H3	22
pAM504	pRL444 derivative lacking *luxAB* and containing *Bam*HI-*Sst*I portion of pUC18 cloning site; Km^r	C. Carrasco
pAM709	235-bp insert containing *xisA* upstream region in pBluescript SK(−); Ap^r	33
pAM684	pAM165 modified by deletion of sequences between *Hin*dIII and *Bam*HI flanking the Ω Sp^r-Sm^r cassette to produce cassette flanked by *Sma*I to *Eco*RI sites of pUC1819H3	This study
pAM996	4.2-kb insert containing *ntcA* in pBluescript SK(+); Ap^r	40
pAM997	1.68-kb *Hpa*I fragment containing *ntcA* from pAM996 in *Sma*I site of pSE380	40
pAM999	*Eco*RI fragment containing *ntcA* from pAM997 in *Eco*RI site of pSE380	40
pAM1318	pAM996 derivative with Ω Sp^r-Sm^r in *Bbs*I site of *ntcA*; Sp^r Ap^r	This study
pAM1320	pAM1318 insert in pRL271; Sp^r Cm^r Em^r	This study
pAM1322	784-bp *Eco*RI-*Hpa*I fragment containing *ntcA* in pCCB110; Km^r	This study
pAM1323	834-bp *Eco*RI-*Afl*II fragment containing *ntcA* in pAM504; Sp^r Km^r	This study
pAn154.2	*Hin*dIII fragment containing *fdxN* element recombination site in pBR322	19
pAn154.3	*Hin*dIII fragment containing *nifH* in pBR322	21
pAn155	*Eco*RI fragment containing *fdxN* element recombination site in pBR322	19
pAn256	*Hin*dIII fragment containing *nifD* element recombination site in pBR322	21
pAn602	*Hin*dIII-*Eco*RI fragment containing the *rbcL* upstream region in pBR322	32
pBluescript SK(+/−)	cloning vector; Ap^r	Stratagene
pCCB110	*Anabaena* shuttle vector containing pIC20R cloning sites; Km^r	5
pCP106	411-bp *Eco*RI-*Hin*dIII fragment containing *glnA* promoter in pUC18	33
pRL444	*Anabaena* shuttle vector containing promoterless *luxAB* genes; Km^r	12
pRL271	*Anabaena* suicide vector containing *sacB*; Cm^r Em^r	2
pUC1819H3	pUC derivative containing inverted cloning sites	22

and streptomycin, 2 μg/ml each for solid medium and 1 μg/ml each for liquid medium.

Inactivation of *ntcA*. The *ntcA* gene was inactivated by using a *sacB*-mediated positive selection for double recombinants (2, 7). The suicide plasmid pAM1320 was constructed in two steps. (i) A *Sma*I fragment containing the Ω Sp^r-Sm^r cassette from pAM684 was inserted into the *Bbs*I site (blunted) in *ntcA* in pAM996 to produce pAM1318. pAM684 was derived from pAM165 (22) by digestion with *Hin*dIII and *Bam*HI, blunting of all fragment ends, ligation, and selection for the Ω Sp^r-Sm^r cassette recloned in the original pUC1819 vector. The Ω Sp^r-Sm^r cassette in pAM684 is flanked by *Sma*I, *Xma*I, *Kpn*I, *Sst*I, and *Eco*RI sites. (ii) An *Xba*I fragment from pAM1318 containing the interrupted *ntcA* gene was cloned into the *Xba*I site of pRL271 (2).

pAM1320 was transferred into PCC 7120 by conjugation, using standard procedures (13, 22). Four independent exconjugants were selected and grown in 50 ml of BG-11(NH_4NO_3) medium containing spectinomycin and streptomycin for 10 days to allow segregation of double recombinants. Single recombinants were screened for segregation of double recombinants that had lost the vector sequences containing the *sacB* gene by their ability to grow in the presence of sucrose. Filaments from approximately 1 ml of fully grown cultures were fragmented by sonication, plated on BG-11(NH_4NO_3) containing spectinomycin, streptomycin, and 8% sucrose, and grown under standard conditions. Colonies appeared in 2 to 3 weeks. A total of 133 colonies were screened for genuine double recombinants on BG-11(NH_4NO_3) plates containing chloramphenicol (10 μg/ml) and erythromycin (5 μg/ml). One colony, strain AMC236, showed the expected phenotype (Sp^r Sm^r Suc^r Cm^s Em^s). Single and double recombinants were confirmed by Southern analysis.

Complementation of the *ntcA* mutant. Two shuttle vectors containing *ntcA* were constructed and used to complement the *ntcA* mutant strain (see Fig. 1A). pAM1322 contains *ntcA* with 87 bp of upstream sequence on a 784-bp *Eco*RI-*Hpa*I fragment in pCCB110. pAM1322 was constructed by first cloning the *Eco*RI insert from pAM999 into the same site of pBluescript SK(+). The insert was then released with *Bam*HI and *Kpn*I and cloned into the same sites of pCCB110. pAM1323 contains *ntcA* with 251 bp of upstream sequence on a 947-bp *Eco*RI-*Afl*II fragment. pAM1323 was constructed by first placing an Ω Sp^r-Sm^r cassette isolated from pAM165 with *Sma*I into the *Eco*RI site (blunted) of pAM996. An *Xho*I-*Afl*II fragment (blunted) was then cloned into the *Sma*I site of pAM504. Shuttle vectors were transferred into recipient strains by conjugation as previously described (13, 22).

Growth rate determination. Although strains were maintained in the presence of appropriate antibiotics, antibiotics were not added to media for growth and heterocyst induction experiments. Growth was monitored spectrophotometrically as the optical density at 750 nm (A_{750}). *Anabaena* cultures grown for 7 to 10 days were used as the inoculum for 100-ml

BG-11($NaNO_3$) or BG-11(NH_4NO_3) cultures. The initial A_{750} of all cultures was approximately 0.02. The cultures were grown on a rotating platform with 80 to 100 microeinsteins of light intensity m^{-2} s^{-1} at 30°C. One-milliliter samples of each culture were removed for A_{750} measurements at the indicated time intervals.

Heterocyst induction. For the general characterization of heterocyst formation, two 100-ml cultures of each strain, PCC 7120, AMC272, AMC236, AMC273, and AMC274, were grown in BG-11(NH_4NO_3) on a rotating platform with 80 to 100 microeinsteins of illumination m^{-2} s^{-1} at 30°C to an A_{750} of approximately 0.5. The mid-growth-phase filaments were then collected at room temperature by centrifugation at 3,000 × *g* for 5 min and induced to form heterocysts in 100 ml of $BG\text{-}11_0$ under the same growth conditions. For AMC236, half of the culture was transferred to BG-11($NaNO_3$). Samples were collected and examined every 12 or 24 h after induction. Photomicrographs were taken with an Olympus BH2 compound microscope using Nomarski (DIC) optics on Kodak Ektachrome 160T color slide film.

Filaments were collected for RNA isolation at 6-h intervals during a large-scale heterocyst induction of the wild-type strain PCC 7120. PCC 7120 vegetative cells from 80 ml of a 7-day-grown BG-11($NaNO_3$) culture were transferred to 8 liters of AA/8 medium containing $NaHCO_3$ (10 mM), NH_4NO_3 (2.5 mM), and MOPS (5 mM; pH 8.0). AA/8 medium and growth conditions were as previously described (21). Growth was monitored by A_{750} and microscopy. The culture was induced to form heterocysts after 4 days of growth (A_{750} of 0.35). The vegetative filaments were collected by centrifugation at 3,000 × *g* for 5 min at room temperature, washed twice with sterile water, and transferred to 8 liters of AA/8 medium containing 10 mM $NaHCO_3$ but lacking NH_4NO_3 and MOPS. At 6-h intervals, filaments from 1.1-liter samples were collected by centrifugation and were frozen at −85°C for subsequent RNA extraction. Filaments were examined microscopically during the induction; proheterocysts were present at 12 h, and mature heterocysts containing polar granules were present at 15 to 16 h after nitrogen stepdown.

Mobility shift assays. Protein extracts and mobility shift assays were performed essentially as previously described (33, 40). Filaments were harvested from late-exponential-growth-phase cultures grown in 100 ml of BG-11(NH_4NO_3). The cells were broken with a Stansted cell disrupter at 12,000 lb/in^2, and a 10-μg sample of protein precipitating between 20 and 60% ammonium sulfate saturation was analyzed. Each protein sample was equilibrated with labeled DNA fragment (10,000 cpm) and analyzed electrophoretically. The DNA probes were a 252-bp *Xba*I-*Hin*dIII fragment containing the *xisA* upstream region from pAM709 and a 35-bp oligonucleotide containing the *glnA* NtcA binding site (33, 40).

DNA rearrangements in AMC236. AMC236 was analyzed for rearrangement of the *nifD* and *fdxN* elements in the presence and absence of combined nitrogen. AMC236 cultures (100 ml) were grown in BG-11(NH_4NO_3) medium to exponential growth phase and then transferred to 100 ml of BG-11(NH_4NO_3), BG-11($NaNO_3$), or $BG\text{-}11_0$. Filaments were harvested after 3 days and genomic DNA was prepared for Southern analysis as previously described (21). The blot containing *Hin*dIII-digested DNA was probed with the *Hin*dIII fragment An256 (21), which contains the *nifH*-proximal recombination site of the *nifD* element. The same blot was stripped and then probed with the *Eco*RI fragment An155 (19), which contains the *nifB*-proximal recombination site of the *fdxN* element.

Southern analysis. DNA extraction and Southern analysis were essentially as previously described (20, 21). DNA was transferred to modified nylon membranes (MagnaCharge; MSI) with 1 M NaCl–50 mM NaOH. DNA probes were labeled with a random primer kit from Boehringer Mannheim and Southern hybridizations were performed as described elsewhere (21).

Northern (RNA) analysis. AMC236 and wild-type PCC 7120 were grown in 100 ml of BG-11(NH_4NO_3) for 5 days (A_{750}, approximately 0.5). RNA was prepared from filaments with an acidic hot phenol method (27). For Northern blot analysis, approximately 5-μg samples of total RNA were denatured with formaldehyde, separated by electrophoresis on a 1.2% formaldehyde-agarose gel (1), and transferred to MagnaCharge membranes (MSI) with 10× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) (35). The blots were hybridized with random primer-labeled probes at 55°C in 50% formamide–5× SSPE–1% sodium dodecyl sulfate (SDS) and washed at 65°C in 0.5× SSPE–0.1% SDS. For high-stringency washes, 0.2× SSPE–0.1% SDS was used.

RNA was prepared from frozen samples of the large-scale induction of wild-type PCC 7120 by centrifugation through 5.7 M CsCl as previously described (20, 23). RNA samples (20 μg) were transferred from formaldehyde-agarose gels to modified nylon membranes and the Northern blots were hybridized with random primer-labeled probes as previously described (21).

Electronic images. Images in Fig. 1, 4, 5, 6, and 7 were prepared by scanning X-ray film autoradiograms on an Apple OneScanner with Ofoto version 2.0 (Light Source Computer Images) at 8 bits and 200 or 300 dots per inch. The images were processed with NIH Image version 1.52 to improve contrast. Canvas version 3.06 (Deneba Systems) was used to prepare the final figures.

RESULTS

Inactivation of the *Anabaena ntcA* gene. The *ntcA* (*bifA*) gene of *Anabaena* sp. strain PCC 7120 was inactivated by a homologous double recombination event that replaced the chromosomal wild-type gene with an interrupted gene introduced on a suicide plasmid. Figure 1A shows the map of the inactivated *ntcA* gene in pAM1320. Inactivation of the chromosomal *ntcA* gene was confirmed by Southern analysis (Fig. 1B). The single recombinant contained both the wild-type *ntcA* gene and the inactivated copy carried on the integrated plasmid pAM1320, while the double recombinant contained only the inactivated *ntcA* gene. In the *Cla*I digest of the double recombinant, a 5.4-kb fragment containing the 2-kb Ω Sp^r-Sm^r cassette replaced the wild-type 3.4-kb fragment. The 6.3-kb fragment in the single recombinant contains the interrupted *ntcA* fragment along with adjacent pAM1320 sequences. The probe hybridized with a flanking 6.0-kb fragment that is unaffected by the recombination in all three strains. In the *Eco*RI digest, the single 6.6-kb *Eco*RI fragment in the wild type was shifted to 8.6 kb in the double recombinant. The single recombinant shows a 6.7-kb fragment that contains the wild-type *ntcA* adjacent to the integrated pAM1320. In the *Hin*dIII digest, the 5.0-kb wild-type fragment was shifted to 7.0 kb in the double recombinant. The single recombinant shows an 8.9-kb band containing the interrupted *ntcA* gene adjacent to the integrated pAM1320 in addition to the wild-type band.

To confirm that the phenotype of AMC236 was due only to the site-directed inactivation of *ntcA*, the AMC236 strain was complemented with the wild-type *ntcA* gene introduced on plasmids. Two plasmids that contain the *ntcA* gene on a conjugal shuttle vector were constructed (Fig. 1A). pAM1322

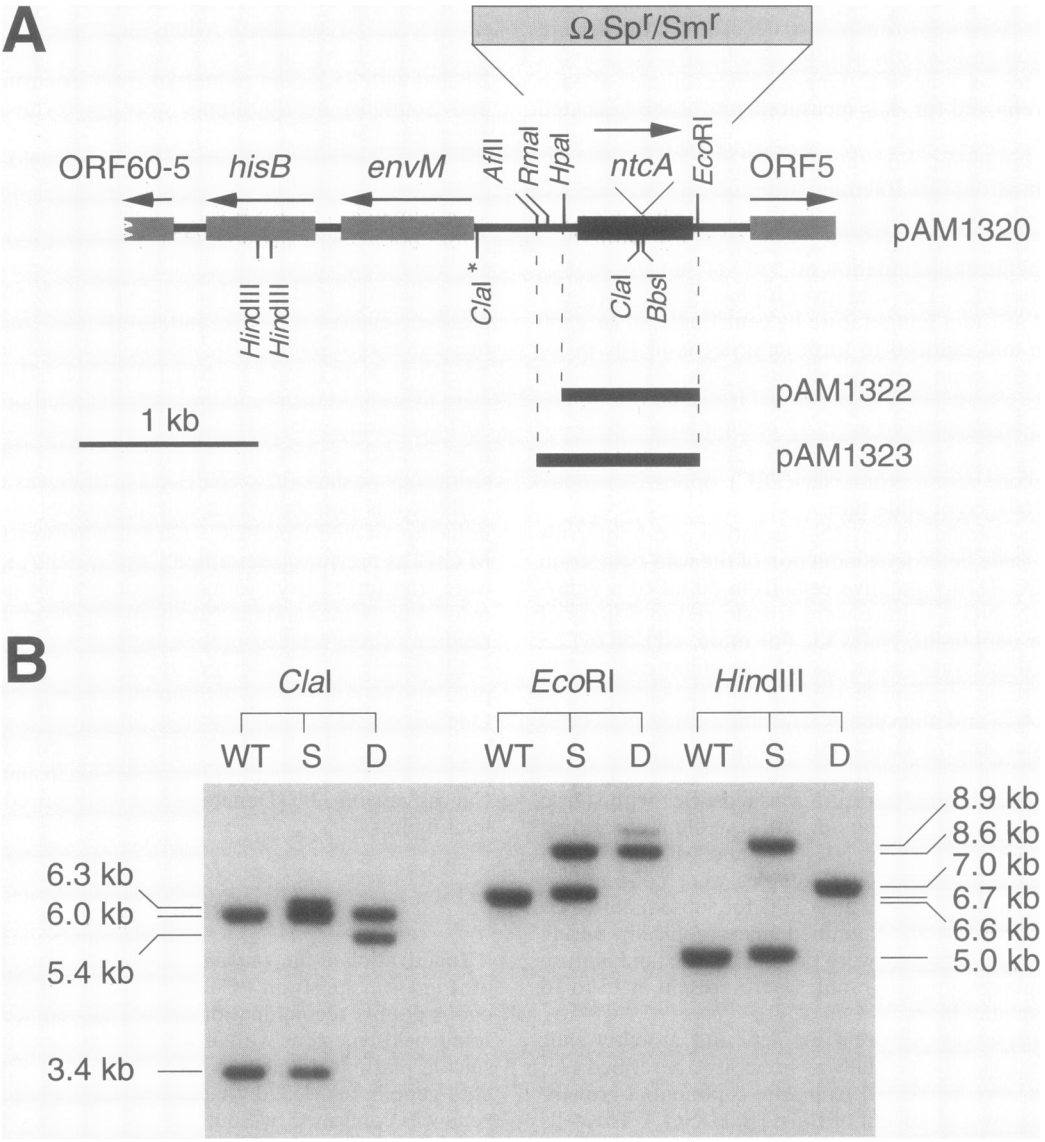

FIG. 1. Map of the *ntcA* region and Southern analysis of the *ntcA* mutant. (A) Map of the pAM1320 insert containing *ntcA*, interrupted by an Ω Sp^r-Sm^r cassette in the *Bbs*I site. ORF60-5 and ORF5 are truncated. Arrows indicate the orientation of gene transcription. The *Cla*I site marked with an asterisk is resistant to cleavage. Fragments used to complement the *ntcA* mutant are shown. pAM1322 contains a 784-bp *Eco*RI-*Hpa*I fragment cloned in pCCB110. pAM1323 contains a 947-bp *Eco*RI-*Afl*II fragment cloned in pAM504. (B) Southern analysis of chromosomal DNA isolated from wild-type PCC 7120 (lanes WT), a single recombinant (lanes S), and a double recombinant (lanes D). Samples digested with *Cla*I, *Eco*RI, or *Hin*dIII were hybridized with a 834-bp *Eco*RI fragment from pAM997 that contains the *ntcA* gene. The faint band seen in *Eco*RI lane D is due to incomplete digestion.

and pAM1323 contain 87 and 251 bp, respectively, of *ntcA* upstream sequence.

***ntcA* mutant requires ammonium for growth.** The growth rate of the *ntcA* mutant AMC236 was compared with those of wild-type PCC 7120 and the two complemented strains, AMC273 and AMC274. Figure 2 shows growth curves for cultures grown in BG-11($NaNO_3$) or BG-11(NH_4NO_3). AMC236 did not grow with nitrate as the sole source of combined nitrogen. Although AMC236 was able to grow in ammonium-containing medium, its growth plateaued at a density half that reached by the wild type. Additional NH_4NO_3 (2 mM) added to a 2-week-old stationary-phase AMC236 culture did not allow continued growth, suggesting that the cessation of growth was not simply due to depletion of ammonium. Both

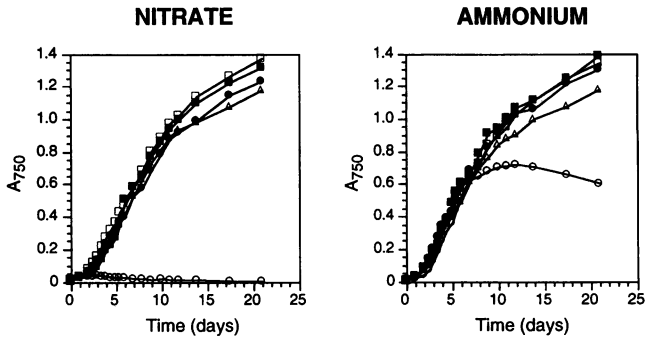

FIG. 2. Growth of the *ntcA* mutant on nitrate, BG-11($NaNO_3$), or ammonium, BG-11(NH_4NO_3), as the nitrogen source. ●, PCC 7120; ■, AMC272; ○, AMC236; △, AMC273; □, AMC274.

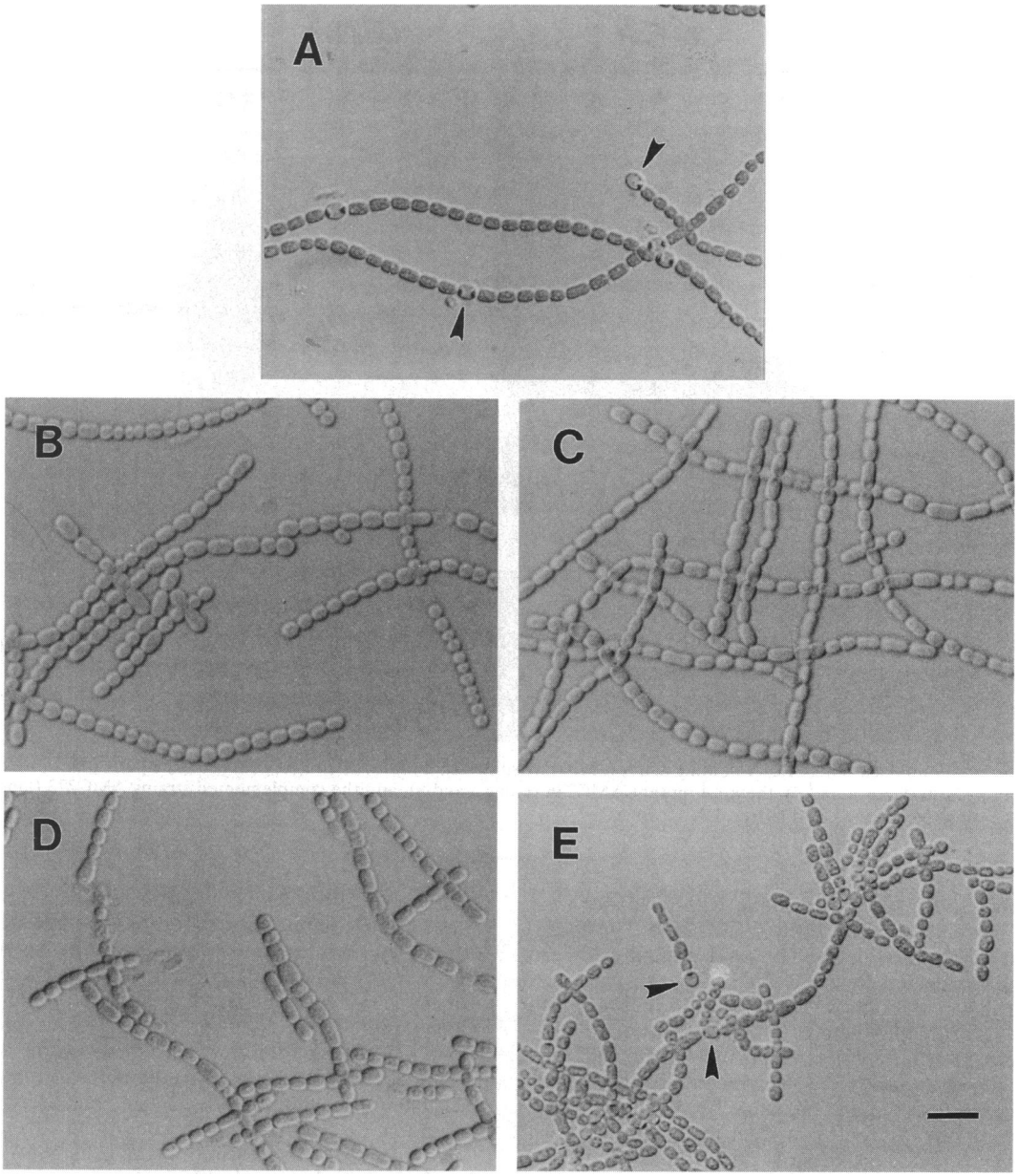

FIG. 3. Light microscopy of induced *Anabaena* cultures. (A) AMC272; (B and C) AMC236; (D) AMC273; (E) AMC274. All cultures except for the one shown in panel C were induced to form heterocysts on nitrogen-free BG-11_0 medium for 3 days. The culture in panel C was grown on BG-11($NaNO_3$) medium for 3 days. Arrowheads indicate heterocysts. Bar, 10 μm.

AMC273 and AMC274 showed growth rates in media containing nitrate or ammonium that were similar to that of the wild type. Therefore, the defect in AMC236 can be attributed solely to the inactivation of *ntcA*.

Extra copies of *ntcA* did not produce a detectable phenotype. AMC272 is the wild-type strain PCC 7120 harboring the shuttle plasmid pAM1323. AMC272 showed normal growth and morphology when grown in media containing nitrate or ammonium (Fig. 2).

***ntcA* is required for heterocyst differentiation.** AMC236 was unable to grow in BG-11_0 medium, which lacks a source of combined nitrogen, and failed to form heterocysts after nitrogen stepdown (Fig. 3B). The appearance of AMC236 filaments in BG-11_0 and BG-11($NaNO_3$) was similar; filaments turned pale, fragmented, and eventually lysed in both media. Although AMC273, which carries the smaller *ntcA* fragment in pAM1322, showed nearly normal growth in the experiment whose results are shown in Fig. 2, it failed to form heterocysts, turned pale, and fragmented in BG-11_0, similarly to AMC236 (Fig. 3D). In contrast, AMC274, which carries the longer *ntcA* fragment in the shuttle vector pAM1323, formed heterocysts at about the same time and frequency as the wild type when transferred to a nitrogen-free medium (Fig. 3E).

Heterocyst formation and frequency appeared normal in AMC272 (Fig. 3A); however, cells of AMC272 and AMC274 were slightly smaller than wild-type PCC 7120 cells.

***ntcA* encodes the DNA-binding factor VF1.** To prove that *ntcA* encodes the DNA-binding factor originally named VF1

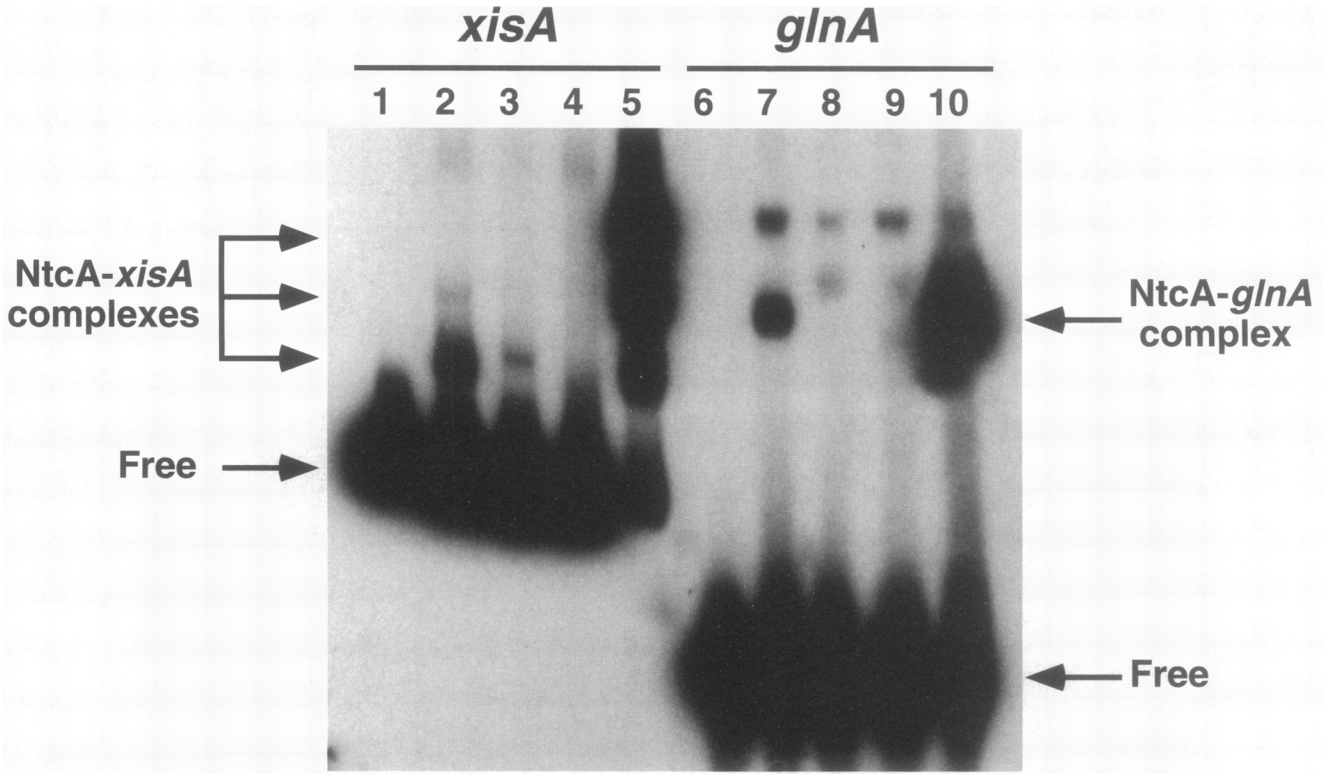

FIG. 4. The *ntcA* mutant lacks NtcA DNA-binding activity. Mobility shift assays with *xisA* and *glnA* probes were performed with protein extracts from wild-type PCC 7120 (lanes 2 and 7), the *ntcA* mutant AMC236 (lanes 3 and 8), and the complemented strains AMC273 (lanes 4 and 9) and AMC274 (lanes 5 and 10). Lanes 1 and 6, free DNA probes.

(9, 40), mobility shift assays were done with *xisA* and *glnA* probes and protein extracts from PCC 7120, AMC236, AMC273, and AMC274 (Fig. 4). The *ntcA* mutant AMC236 did not show the expected complexes with either probe. NtcA DNA-binding activity not only was restored in AMC274, which had wild-type growth and heterocyst differentiation phenotypes, but was significantly greater than that of the wild type (Fig. 4, lanes 5 and 10). AMC273, which contains the shorter *ntcA* fragment, did not contain detectable NtcA DNA-binding activity in this in vitro assay. We suspect that AMC273 must have a low level of *ntcA* expression since it showed partial complementation of the AMC236 phenotype and grew nearly as well as the wild type on ammonium- and nitrate-containing media.

***nifD* and *fdxN* rearrangements.** NtcA binds to three sites upstream of the *xisA* gene near a region that suppressed read-through transcription of *xisA*, suggesting a possible regulatory function of NtcA binding on *xisA* expression (4, 9). Excision of the *nifD* and *fdxN* elements was examined by Southern analysis of DNA isolated from AMC236 grown on BG-11(NH_4NO_3) and after transfer to BG-11($NaNO_3$) or BG-11_0 (Fig. 5). DNA isolated from wild-type vegetative cells and purified heterocysts is shown for comparison. The *Hin*dIII fragment An256, which spans one of the *nifD* element recombination sites (20), hybridized with a 2.9-kb band in wild-type vegetative cell DNA and with 2.1- and 1.8-kb bands in heterocyst DNA. The *Eco*RI fragment An155, which spans one of the *fdxN* element recombination sites (19), hybridized with a 3.6-kb band in wild-type vegetative cell DNA and with 4.6- and 2.2-kb bands in heterocyst DNA. AMC236 did not rearrange the *nifD* element or *fdxN* element under any of these growth conditions. This is not very surprising, since the *ntcA* mutant does not form heterocysts. After longer exposures, a low level of *fdxN* element excision was detected in the AMC236 BG-11($NaNO_3$)- and BG-11_0-grown cultures. This result was confirmed by hybridizing the blot with the An154.2 fragment, which contains the other end of the *fdxN* element (data not shown). The significance of this observation is not known, although it could be related to the detection of *fdxN* element excision in the absence of *nifD* element excision under microaerobic heterocyst-inducing conditions (18).

***glnA* and *rbcLS* expression in AMC236.** NtcA interacts with the *glnA*, *xisA*, *rbcL*, and *nifH* upstream regions in vitro (33). Expression of the heterocyst-specific *xisA* and *nifH* genes was not examined because AMC236 does not form heterocysts and because *xisA* expression is difficult to detect (4). However, *xisA* expression was indirectly monitored by assaying the *nifD* rearrangement, as described in the previous paragraph. Northern analysis of *glnA* and *rbcLS* messages in total RNA isolated from wild-type PCC 7120 and AMC236 grown in BG-11(NH_4NO_3) showed an interesting change in the pattern of *glnA* expression (Fig. 6). Two major *glnA* messages of 1.9 and 1.7 kb were observed in wild-type PCC 7120. In AMC236, however, the total amount of *glnA* message was reduced and, most strikingly, the 1.7-kb message was absent. This suggests that *ntcA* may be required for the expression of the 1.7-kb message, which is the major message present during heterocyst induction (see below). An additional message of approximately 1.8 kb that is difficult to observe in the wild type because of the strong 1.7- and 1.9-kb bands could be seen in AMC236.

Analysis of *rbcLS* messages does not show any significant differences between AMC236 and wild-type PCC 7120 under

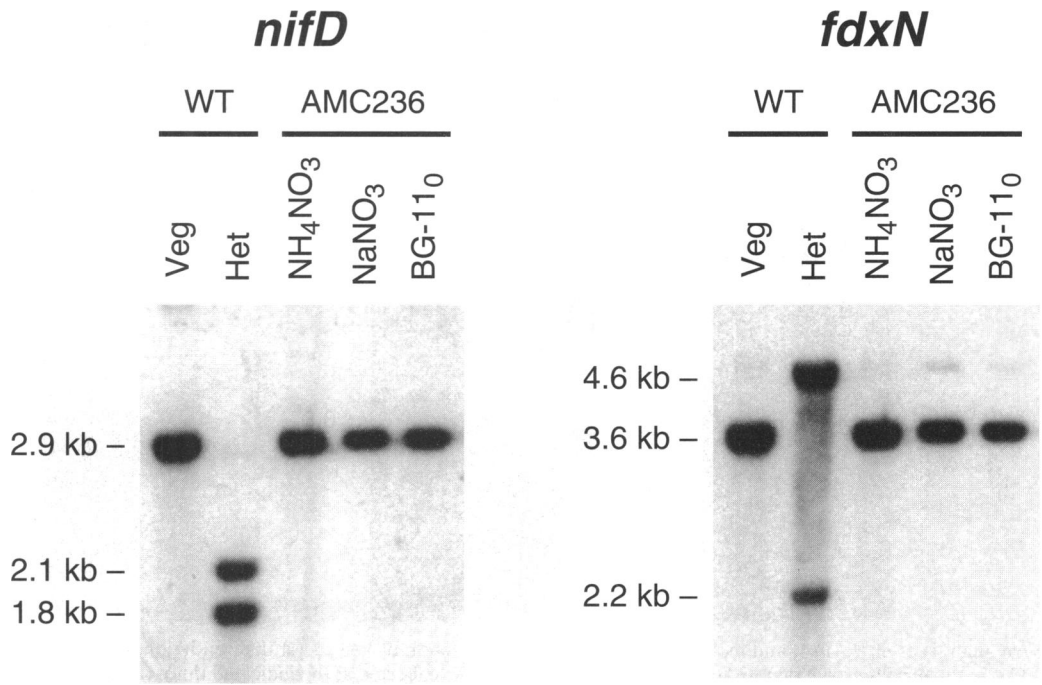

FIG. 5. *ntcA* mutant strain AMC236 fails to rearrange the *nifD* and *fdxN* elements. DNA was isolated from wild-type (WT) vegetative cells (Veg) and purified heterocysts (Het) and from AMC236 grown on BG-11(NH_4NO_3) or after transfer to BG-11($NaNO_3$) or BG-11_0. The DNA samples were digested with *Hin*dIII and used to prepare Southern blots. The blots were hybridized with the DNA fragment An256 to detect excision of the *nifD* element or with An155 to detect excision of the *fdxN* element.

the growth conditions in this study (Fig. 6). This does not exclude the possibility that *ntcA* is involved in the expression of *rbcLS* under different growth or environmental conditions.

***ntcA* expression during heterocyst induction.** The expression of *ntcA* in filaments during heterocyst induction was examined by Northern analysis (Fig. 7). The *nifHDK* operon is expressed late during heterocyst development, at about the same time that mature heterocysts are first observed (14, 21). In the induction shown in Fig. 7, *nifHDK* message was absent from vegetative cells and at early times (6- and 12-h samples) during heterocyst induction and was detected at 18 h after nitrogen stepdown and later. Mature heterocysts were observed in the culture between 15 and 16 h after induction.

ntcA showed an interesting pattern of expression during heterocyst induction (Fig. 7). *ntcA* messages appeared as a smear between 1.4 and 0.8 kb in vegetative cell RNA. The *ntcA* open reading frame is 672 bp (40). During induction, there was a peak of *ntcA* expression at 12 h and then a drop at 18 h followed by a gradual increase in expression at later times. Only the 0.8-kb message was present during the induction. The strong expression of *ntcA* during the early stages of heterocyst differentiation is presumably related to the Het^- phenotype of the *ntcA* mutant.

Expression of *glnA* during heterocyst induction was examined by Northern analysis (Fig. 7). Similar to the results shown in Fig. 6, 1.7- and 1.9-kb *glnA* messages were present in vegetative cells. *glnA* expression dropped off during the early stages of heterocyst induction and then gradually increased later. In contrast to the findings for vegetative cells, only the 1.7-kb message, which was absent in the *ntcA* mutant, was present during the induction up to the last time point, 36 h.

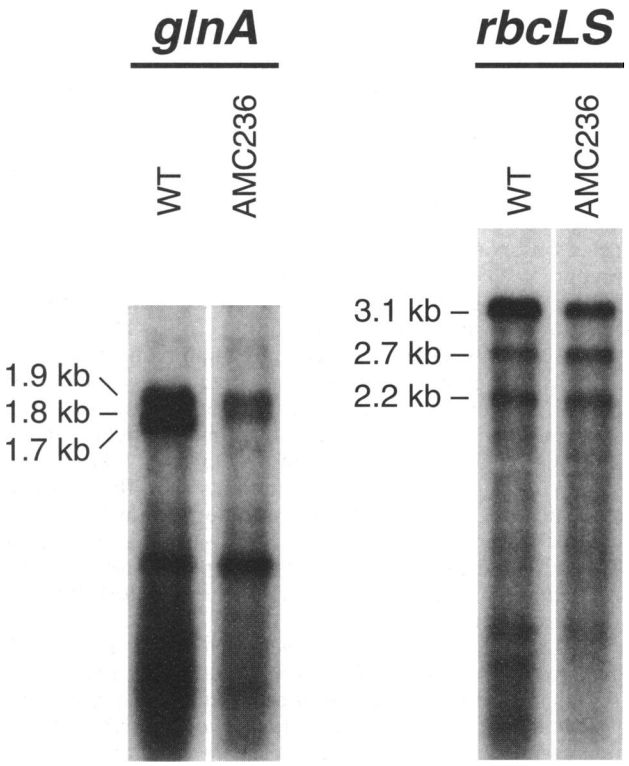

FIG. 6. Northern analysis of *glnA* and *rbcLS* transcripts in RNA isolated from PCC 7120 (WT) and the *ntcA* mutant strain AMC236. Hybridization probes: *glnA*, *Hin*dIII-*Xba*I fragment from pCP106; *rbcLS*, *Eco*RI-*Hin*dIII fragment from pAn602.

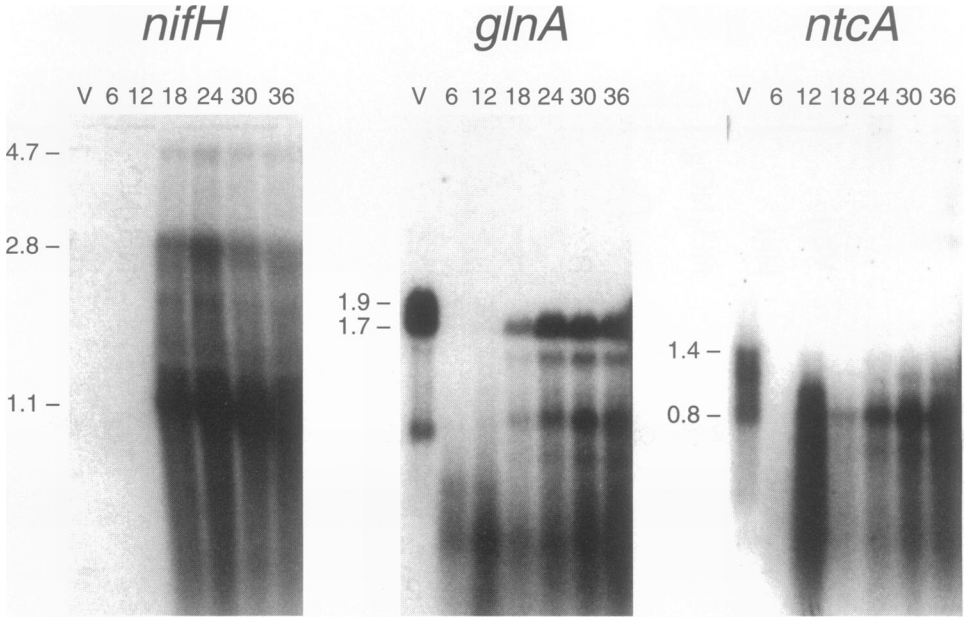

FIG. 7. Northern analysis of *nifH*, *glnA*, and *ntcA* transcripts during the induction of heterocyst differentiation. Total RNA was isolated from wild-type PCC 7120 vegetative cells grown on nitrate (lanes V) and from filaments collected at the indicated times (hours) after nitrogen stepdown. Hybridization probes: *nifH*, *Hin*dIII fragment from pAn154.3; *glnA*, *Hin*dIII-*Xba*I fragment from pCP106; *ntcA*, *Eco*RI fragment from pAM997. Sizes (in kilobases) are indicated to the left of each panel.

DISCUSSION

NtcA (BifA, VF1) was originally identified and characterized as a sequence-specific DNA-binding protein that interacted with the *Anabaena* sp. strain PCC 7120 *xisA* gene, which encodes a developmentally regulated site-specific recombinase (9). NtcA was shown to also interact with several other genes in PCC 7120, including *glnA*, *rbcL*, and *nifH* (33). This information was used to clone the *ntcA* (*bifA*) gene (40). In this study, a reverse genetics approach was used to study the role of NtcA in PCC 7120. Analysis of the *ntcA* mutant strain AMC236 confirmed that *ntcA* encodes the previously characterized DNA-binding protein VF1 and produced the unexpected observation that *ntcA* is required for heterocyst development.

NtcA belongs to the family of bacterial transcriptional regulators of which Crp is the prototype (40). The predicted PCC 7120 NtcA amino acid sequence shows a high degree of similarity to that of NtcA in *Synechococcus* sp. strain PCC 7942 (17, 40), which is required for full expression of nitrate reductase, nitrite reductase, and glutamine synthetase and for methylammonium and nitrate transport (37, 38). Like *Synechococcus* sp. strain PCC 7942 *ntcA* mutants (38), the *Anabaena* sp. strain PCC 7120 *ntcA* mutant required ammonium for growth. However, in PCC 7120, NtcA's in vitro interactions with *rbcLS* and *xisA* (9, 33) and its requirement for heterocyst differentiation suggest that it may have regulatory functions other than nitrogen control.

A high level of glutamine synthetase activity is required for cells growing on limiting ammonium, and under these conditions glutamine is an essential intermediate in the synthesis of all cellular nitrogen compounds (26). *glnA* expression in several cyanobacteria has been partially characterized. In *Anabaena* sp. strain PCC 7120, *glnA* is transcribed from multiple promoters that are differentially expressed in response to changes in nitrogen availability (36). The amino acid sequence of the glutamine synthetase in the filamentous nitrogen-fixing cyanobacterium *Calothrix* sp. strain PCC 7601 is 89% identical to that of the PCC 7120 protein, and the regions upstream of the *glnA* genes in these two organisms are 74% identical (15). Two *glnA* messages are present in PCC 7601: a 1.6-kb message that predominates in nitrate-grown cells and a 1.8-kb message that predominates in ammonium-grown cells (15). Transcripts from the *Synechococcus* sp. strain PCC 7002 *glnA* gene increase three- to fivefold when the cells are starved for nitrogen, and a putative NtcA binding site is located upstream of the major transcription start site (39). The *Synechococcus* sp. strain PCC 7942 *glnA* gene is expressed from a single *nif*-like promoter and showed reduced levels of transcripts when ammonium was present in the medium (10). The transcription start site was identified at −146 nucleotides upstream of the open reading frame, and a putative NtcA binding site was found −35 to −51 nucleotides upstream of the transcription start site (10).

NtcA may be the nitrogen sensor that mediates the differential expression of *glnA* in PCC 7120. A 1.7-kb *glnA* transcript was absent in the *ntcA* mutant yet highly expressed in the wild type grown under the same conditions. During a heterocyst induction, *glnA* was expressed predominantly as the 1.7-kb transcript. The expression pattern of the 1.7-kb band during nitrogen stepdown corresponds with the transcription of RNA_I in ammonium-starved cells observed by Tumer et al. (36). It is likely that the 1.7-kb message corresponds to transcription from RNA_I. We propose that NtcA acts as an activator of RNA_I transcription. This is consistent with our previous studies that identified an NtcA binding site between the *glnA* RNA_I and RNA_{II} promoters (33) in a position typical for an activator of the RNA_I promoter and for a repressor of the RNA_{II} promoter (33). It is likely that the PCC 7601 1.6-kb message requires NtcA for expression, because the upstream region of the PCC 7601 *glnA* gene contains a 21-bp sequence that is identical to the PCC 7120 NtcA binding site (15, 33).

The role of NtcA binding to the upstream region of *xisA* is not known. The failure of the *ntcA* mutant to rearrange the *nifD* and *fdxN* elements can be attributed to the early block in heterocyst differentiation. Since forced expression of *xisA* in vegetative cells is sufficient to cause excision of the *nifD* element (4), the lack of *nifD* rearrangement in AMC236 shows that NtcA is not essential for the repression of *xisA* in vegetative cells. The NtcA binding site upstream of *xisA* is centered about 65 nucleotides from a recombination site for the *nifD* rearrangement. NtcA could be involved in the site-specific recombination reaction by bending the DNA in a manner similar to that of integration host factor (IHF) and factor for inversion stimulation (Fis) (16, 25).

No significant changes in *rbcLS* messages were evident in AMC236 grown under our standard conditions. In vitro footprint analysis identified an NtcA binding site encompassing the *rbcL* transcription start site, a position typical of transcriptional repressors (11, 33). It is possible that under some growth conditions, such as high CO_2 concentrations, NtcA may be involved in negatively regulating *rbcLS* transcription.

It is not known why heterocyst differentiation is blocked in the *ntcA* mutant. Altered expression of the genes that NtcA is known to interact with would not be expected to produce an early block in heterocyst development. We speculate that the burst of *ntcA* expression early during heterocyst induction is indicative of *ntcA*'s involvement in the regulation of genes, such as *hetR* (5), that are required for heterocyst development. It should be emphasized that our analysis of RNA isolated from whole filaments does not provide information about gene expression in different cell types; we do not know if the increased *ntcA* expression at 12 h is localized to a particular cell type.

The *ntcA* mutant was fully complemented by the *ntcA* fragment containing 251 bp of upstream sequence in pAM1323 but was only partially complemented by the fragment containing 88 bp of upstream sequence in pAM1322. This result suggests that sequences required for full expression of *ntcA* are present between −88 and −251 bp.

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