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Localized Induction of the *ntcA* Regulatory Gene in Developing Heterocysts of *Anabaena* sp. Strain PCC 7120

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The *ntcA* gene encodes an N-control transcriptional regulator in cyanobacteria. In the N_2-fixing, heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120, *ntcA* is an autoregulatory gene that is transcribed from a complex promoter region that includes a constitutive promoter (P_2) and promoters that are induced upon N step-down (P_1 and P_3). Expression of *ntcA* was investigated with the use of an *ntcA-gfp* translational fusion, which was introduced both in the natural *ntcA* locus and in a heterologous genomic place. Induction of *ntcA-gfp* took place after N step-down in all the cells of the filament, but at especially high levels in developing heterocysts. Localized induction could be driven independently by P_3 and P_1.

Some filamentous cyanobacteria, such as those of the genera *Anabaena* and *Nostoc*, develop heterocysts, which are cells specialized in N_2 fixation (9, 11, 17, 31). The initiation of heterocyst differentiation is dependent on the NtcA transcription factor, which is a global regulator of nitrogen assimilation (8, 10, 11, 30), as well as on other early acting developmental regulatory genes, such as *hetR* (3, 4). The *hetR* gene is induced after N step-down in an NtcA-dependent manner (8, 21). The *ntcA* gene is autoregulatory, and induction of *ntcA* in *Anabaena* sp. strain PCC 7120 takes place after N step-down (21, 24). NtcA binds to DNA sites bearing the sequence signature $GTAN_8TAC$ (10, 13), and the activity of NtcA as a transcriptional regulator is positively modulated by 2-oxoglutarate, a metabolite that lies at the link of C and N metabolisms in cyanobacteria (12, 14, 22, 26, 28, 29). Induction of *ntcA* also requires the action of HetR (21). Although HetR dependency is suggestive of induction in heterocysts or developing heterocysts (proheterocysts), localized induction has not been demonstrated for *ntcA*.

The *ntcA* gene is transcribed from a complex promoter region that gives rise to three transcription start points (TSPs) (21, 24). Promoter P_1 produces a TSP at −49 (that is, 49 nucleotides upstream from the translational start of *ntcA*), P_2 produces a TSP at −136, and P_3 produces a TSP at −180. P_2 functions constitutively, i.e., independent of the N source. However, an NtcA binding site of sequence $GTAN_8AAC$, which is centered at −143.5 and has been footprinted with DNase I (24), overlaps the −10 hexamer of this promoter (21). P_1 also functions in filaments grown with different N sources, but its utilization increases after 6 to 12 h of N deprivation in an NtcA- and HetR-dependent manner, and it is very strong in heterocysts. P_3 functions only in the absence of combined N, being transiently induced after 6 to 12 h of N deprivation in an NtcA- and HetR-dependent manner (21). A second putative NtcA binding site, which exhibits a perfect NtcA sequence signature, $GTAN_8TAC$, is found in the *ntcA* promoter region centered at −103.5. Although this NtcA binding site did not show up in DNase I footprinting analysis (24), two retarded bands have been observed in band shift assays with NtcA and a DNA fragment of the *Anabaena ntcA* promoter region (12). To characterize the contribution of different elements of this promoter region to the expression of *ntcA* and the pattern of *ntcA* expression along the filament, we prepared an *ntcA-gfp* translational fusion and analyzed its expression in constructs that carried different portions of the *ntcA* promoter region.

Methods. *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 and its derivatives were grown photoautotrophically at 30°C in light (75 $\mu E \cdot m^{-2} \cdot s^{-1}$) in $BG11_0C$ medium (BG11 medium [25] without $NaNO_3$ and supplemented with 0.84 g · $liter^{-1}$ of $NaHCO_3$) supplemented with 6 mM NH_4Cl plus 12 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH buffer (pH 7.5), bubbled with a mixture of CO_2 and air (1% vol/vol), and supplemented with 2 $\mu g \cdot ml^{-1}$ of streptomycin and 2 $\mu g \cdot ml^{-1}$ of spectinomycin in the case of the CSEL strains. Molecular biology methods were standard (1).

To determine green fluorescent protein (GFP) fluorescence levels and for isolation of RNA, cells growing exponentially in $BG11_0C$ medium supplemented with NH_4Cl (cultures with about 4 μg of chlorophyll *a* per ml; chlorophyll *a* levels were determined with methanolic extracts [15]) were harvested at room temperature and either used directly or washed with $BG11_0C$ medium, resuspended in $BG11_0C$ (nitrogen-free) medium, and further incubated under culture conditions for the number of hours indicated for each experiment in the figure legends. The accumulation of GFP reporter was analyzed by laser confocal microscopy as described previously (19). All confocal images for each experiment were collected using the same settings so that the intensities could be compared. Total RNA from cyanobacteria was isolated (21) and primer extension analysis was performed (20) as previously described.

Construction of *Anabaena* strains carrying the *ntcA-gfp* fusion. The *ntcA* promoter region (positions −645 to +24 with respect to the translational start of *ntcA*) was PCR amplified using oligonucleotides NA13 and NA14 (introducing an EcoRV site centered at position +18.5) (Table 1) and plasmid

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TABLE 1. Deoxyoligonucleotides used in this work[a]

Primer	Sequence (5′ to 3′)
NA13	CCAGTCAGATTCAGCATAG
NA14	GGC**GA**TATC**T**TGTGTCACG
NA15	GAAGTATC**GAT**GAAGAACTGC
NA16	AGTTGGGTATC**GAT**ATGAAC
NA17	GT**A**TCGATAGGTTAACGGTGC
NUI3	AGCTGTCAATGTCTACCAC
GFP3	GAATTGGGACAACTCCAGTG
GFP4	CAAGAATTGGGACAACTCC

[a] Restriction endonuclease recognition sites are underlined, and nucleotides changed are indicated in boldface type.

pCSE4 (8) as a template and cloned into vector pGEM-T (Promega), rendering pCSEL18. Because the ClaI site centered at position −600.5 is affected by *dam* methylation, pCSEL18 was transformed into *Escherichia coli* strain GM48 (23). A ClaI-EcoRV fragment from pCSEL18, containing the complete *ntcA* promoter, was cloned into ClaI-EcoRV-digested pIC20R (16), rendering pCSEL20. An EcoRV-PstI fragment from pCSEL19 (19) containing a promoterless *gfp* gene was inserted between EcoRV and PstI restriction sites in pCSEL20 downstream from the *ntcA* promoter, rendering plasmid pCSEL21. This plasmid contains a translational fusion between the *ntcA* promoter region (plus sequences encoding the first six amino acids of NtcA) and promoterless *gfp* (see Fig. 1A). The EcoRI fragment from pCSEL21 containing P*ntcA-gfp* was cloned into EcoRI-digested pCSV3 (which is a derivative of mobilizable vector pRL500 [6] in which the Ap^r gene has been excised with DraI and replaced by the DraI-ended Sm^r Sp^r cassette C.S3 [6] in the same orientation; V. Rodríguez, unpublished), rendering pCSEL22a. This plasmid can be integrated upon homologous recombination in the *ntcA* region of *Anabaena* sp. strain PCC 7120 (see below).

To prepare constructs bearing deleted versions of the *ntcA* promoter, different fragments from the *ntcA* upstream region were amplified by PCR using oligonucleotides NA14 (see above) and NA15, NA16, or NA17 (Table 1) and plasmid pCSE4 (8) as a template. Oligonucleotides NA15, NA16, and NA17 introduce ClaI restriction sites (Table 1). The PCR products (see Fig. 2) were cloned in pGEM-T or pGEM-T Easy (Promega). Plasmids pCSEL30, pCSEL36, and pCSEL37 were prepared by replacing the ClaI-EcoRV fragment from pCSEL21 with ClaI-EcoRV fragments corresponding to NA14-NA15, NA14-NA16, and NA14-NA17, respectively.

Mobilizable plasmid pCSEL24 was prepared to integrate the constructs described above into a neutral site (*nucA-nuiA* region) located in the α megaplasmid of *Anabaena* sp. strain PCC 7120. Plasmid pCSEL24 consists of pCSAV80 (19), a derivative of pCSAM28 (18), bearing a BamHI-ended Sm^r Sp^r C.S3 inserted into the BamHI site of the *bla* gene. EcoRI-PstI fragments containing *ntcA-gfp* fusions from plasmids pCSEL21, pCSEL30, pCSEL36, and pCSEL37 (see above) were cloned into EcoRI-PstI-digested pCSEL24, rendering pCSEL27, pCSEL31, pCSEL38, and pCSEL39, respectively. As a control, a construct containing promoterless *gfp* was prepared. An EcoRV-PstI fragment from pCSEL19 (19) containing a promoterless *gfp* was inserted between EcoRV and PstI restriction sites in pIC20R (16), rendering plasmid pCSEL50. Fragment EcoRI-PstI from plasmid pCSEL50 containing the promoterless *gfp* was cloned into EcoRI-PstI-digested pCSEL24 (see above), rendering pCSEL51.

Plasmids bearing the *ntcA-gfp* fusion or promoterless *gfp* were transferred to *Anabaena* sp. strain PCC 7120 by conjugation (7), using helper plasmid pRL623 (5), to generate strains bearing the *ntcA-gfp* translational fusion in the *ntcA* genomic region (pCSEL22a for strain CSEL4a) or in the *nuiA-nucA* region (pCSEL27, pCSEL31, pCSEL38, and pCSEL39 for strains CSEL6, CSEL7, CSEL8, and CSEL9, respectively). Promoterless *gfp* was introduced in the *nuiA-nucA* region (pCSEL51, strain CSEL13). Exconjugants were selected in $BG11_0$ medium supplemented with streptomycin and spectinomycin, and their genomic structure was confirmed by Southern analysis. Additionally, the modified *ntcA* promoter regions introduced in the *nuiA-nucA* region were PCR amplified using oligonucleotides NUI3 and GFP4 and DNA from the corresponding *Anabaena* strains as template, and the PCR products were sequenced.

Localized induction of *ntcA* during heterocyst differentiation. Upon conjugation from *E. coli* to *Anabaena* sp. strain PCC 7120, plasmid pCSEL22a, which carries 603 bp from the region upstream of the *Anabaena ntcA* gene, was integrated, resulting in a duplication of the *ntcA* promoter region (Fig. 1B). In the resulting strain, CSEL4a, one copy of the promoter directed expression of wild-type *ntcA*, whereas the other copy directed expression of the *ntcA-gfp* fusion. Construct in pCSEL22a was also obtained with the insert in the opposite orientation, rendering pCSEL22b, which was also conjugated into *Anabaena* sp. strain PCC 7120, rendering strain CSEL4b. The two orientations resulted in similar expression and, thus, only results obtained with CSEL4a are shown below.

A faint green fluorescence was observed for ammonium-grown filaments of strain CSEL4a (Fig. 1C). Green fluorescence levels were increased in all cells of the filaments incubated for 12 to 24 h in the absence of a source of combined N (Fig. 1C), indicating an increased production of GFP. However, as early as 6 h after N step-down, some cells of the filament showed higher GFP fluorescence levels than their neighboring cells (Fig. 1C). Based on morphology and staining with Alcian blue (cell suspension mixed 1:1 with a 1% Alcian blue [Sigma] solution; not shown), the cells overexpressing GFP at 6 and 12 h could be identified as proheterocysts. These results showed that the increased expression of *ntcA* that takes place in the filaments of *Anabaena* sp. strain PCC 7120 after N step-down is localized, to a significant extent, to developing heterocysts.

The expression of *ntcA* also took place at a high level in fully developed heterocysts, which showed a decreased autofluorescence from photosynthetic pigments, such as those observed at 24 h of incubation (Fig. 1C). However, some heterocysts, presumably older ones, that can be found in steady-state N_2-fixing cultures of strain CSEL4a showed a decreased GFP fluorescence (not shown). These results suggest that an increased *ntcA* expression takes place specifically in developing and mature heterocysts, at least up to a certain heterocyst age.

GFP expression from altered versions of the *ntcA* promoter region. To identify which of the *ntcA* promoters contribute to induction of *ntcA* in the proheterocysts, three deletions, covering different lengths from the distal (5′) promoter region,

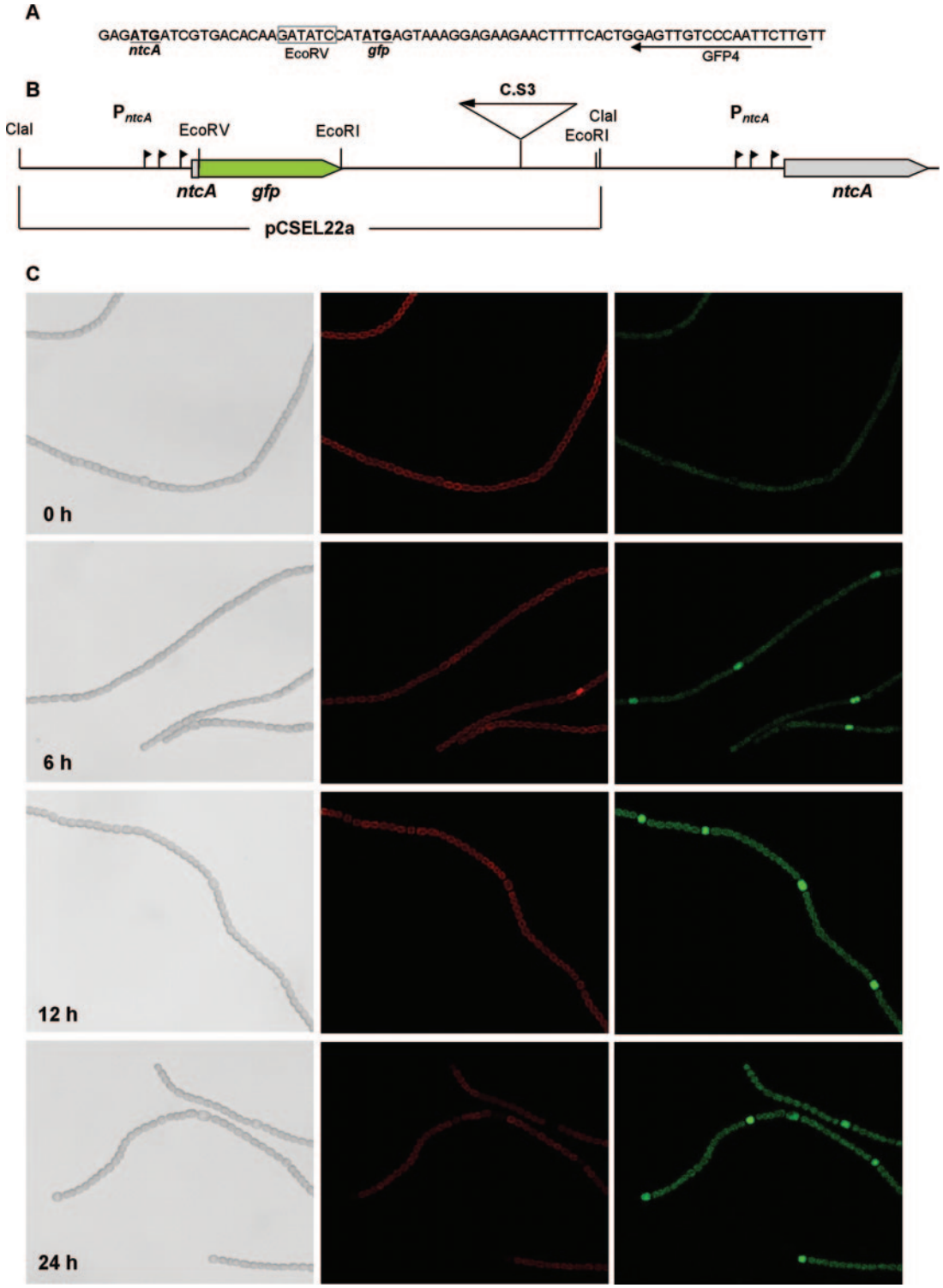

FIG. 1. Structure and expression of the *ntcA-gfp* fusion integrated in the *ntcA* locus. (A) Nucleotide sequence showing the *ntcA* and *gfp* start codons (underlined) and illustrating the *ntcA-gfp* fusion. The locations of a relevant EcoRV restriction site and the GFP4 oligonucleotide are also indicated. (B) Scheme of the *ntcA* genomic region in strain CSEL4a, which carries pCSEL22a integrated in the *Anabaena* chromosome. Flags indicate the approximate location of the *ntcA* transcription start points. Relevant restriction sites are also indicated. (C) Light transmission micrographs (left column), phycobiliprotein autofluorescence (middle column), and GFP fluorescence (right column) of filaments of strain CSEL4a from an ammonium-grown culture (0 h) or from ammonium-grown cultures incubated for 6, 12, or 24 h in a medium lacking combined nitrogen.

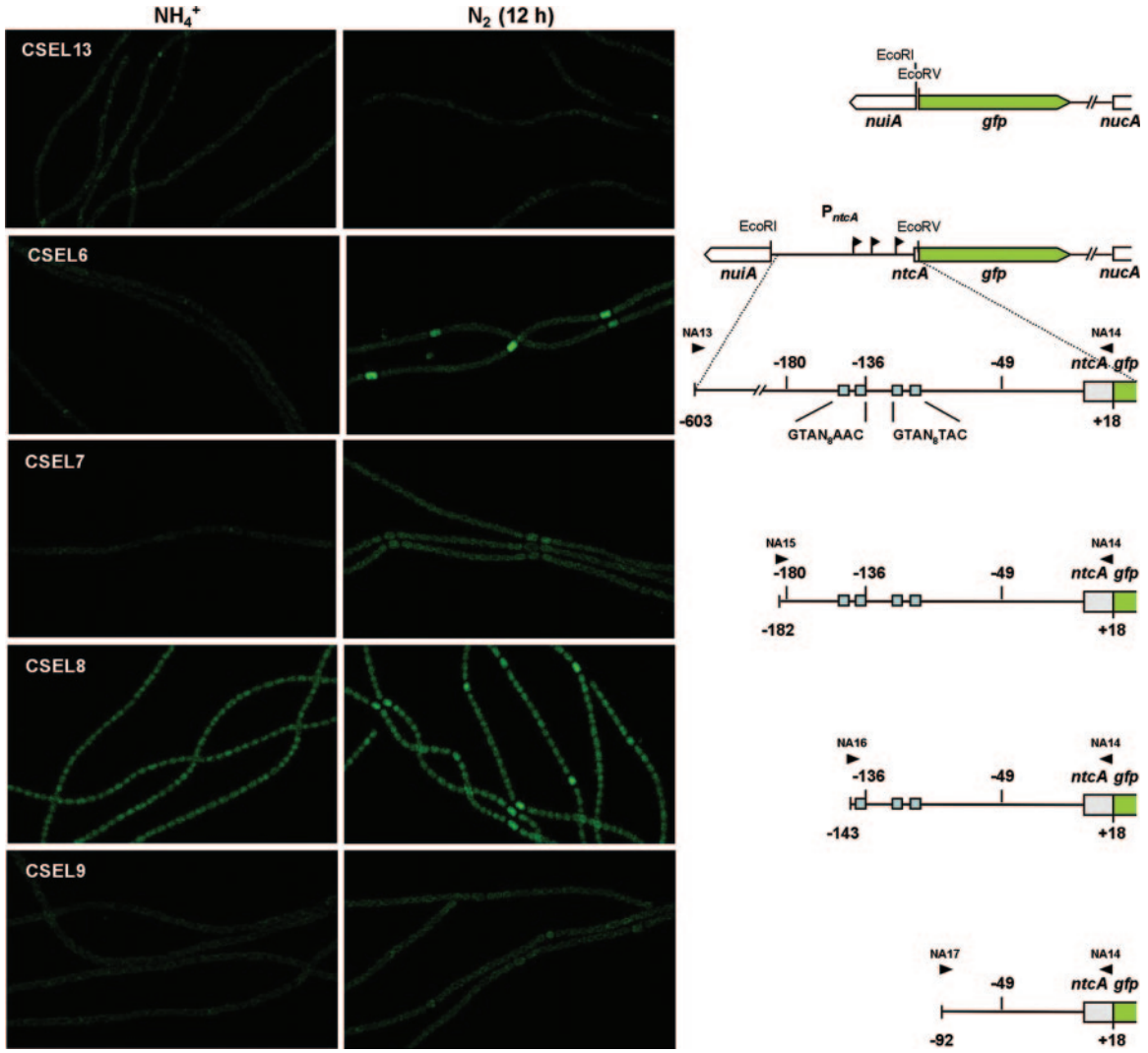

FIG. 2. Schematic representation of the altered versions of the *ntcA* promoter region and GFP fluorescence of strains carrying the *ntcA-gfp* fusions or a promoterless *gfp* gene integrated in the *nuiA-nucA* genomic site. GFP micrographs of filaments from ammonium-grown cultures or from ammonium-grown cultures incubated for 12 h in a medium lacking combined nitrogen are shown for the indicated CSEL strains. In the schemes, the structure of the *nuiA-nucA* region is outlined for strains CSEL13 and CSEL6, and the approximate locations of the −49, −136, and −180 TSPs, of the two NtcA binding sites, and of relevant deoxyoligonucleotide primers are indicated for strains CSEL6 through CSEL9.

were prepared (Fig. 2). Because altered versions of the *ntcA* promoter could alter expression of the wild-type *ntcA* gene if integrated in the *ntcA* locus of the genome, all of these constructs were integrated, through homologous recombination, in a neutral site of the genome (the *nuiA-nucA* region of the *Anabaena* α megaplasmid). A wild-type version bearing the same fusion as that contained in pCSEL22a was integrated in the neutral site, rendering strain CSEL6, which was used as a positive control. As a negative control, a promoterless *gfp* gene was integrated in the same site, rendering strain CSEL13. A very low level of green fluorescence was observed for strain CSEL13; it did not increase after incubation in the absence of ammonium (Fig. 2). Strain CSEL6 also showed a basal level of green fluorescence in ammonium-grown filaments but, in this case, an increased GFP fluorescence was observed for all the cells of the filament after N step-down (Fig. 2). As described above for strain CSEL4a, a higher GFP fluorescence was localized to specific cells in strain CSEL6. These observations indicate that expression of the *ntcA-gfp* fusion was not altered by integration into the heterologous *nuiA-nucA* locus.

In strain CSEL7, which lacks *ntcA* promoter sequences upstream from position −182, GFP fluorescence was low in ammonium-grown cells and increased in all the cells of the filament upon N step-down, but a higher induction localized to specific cells was not observed (Fig. 2). Thus, DNA upstream from nucleotide −182 appears to be essential for localized induction of *ntcA* in proheterocysts. On the other hand, the deletion of DNA upstream from nucleotide −143 (strain CSEL8) resulted in GFP fluorescence that was higher in ammonium-grown filaments than in the control strain CSEL6 or in strain CSEL7 (Fig. 2). This result suggests that, at least in the absence of sequences upstream of −182, the DNA between positions −143 and −182 exerts a negative role in expression. Additionally, after N step-down, induction of GFP fluores-

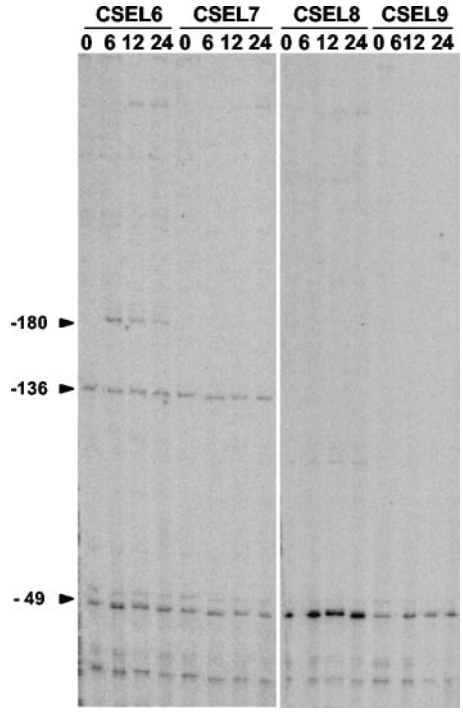

FIG. 3. Primer extension analysis of the expression of the *ntcA-gfp* fusion. Primer extension was carried out with primer GFP4 (Fig. 1A), and RNA was isolated from filaments grown with ammonium, washed, and incubated for 6, 12, and 24 h in the absence of combined nitrogen.

cence was observed for specific cells in the filaments of strain CSEL8. The deletion of DNA upstream from position −92 (strain CSEL9) resulted in production of low levels of GFP fluorescence, although an increase in fluorescence was observed after N step-down in all the cells of the filament. This result indicates a positive role of the DNA between positions −92 and −143, which contains the −103.5 NtcA binding site, in expression of the fusion.

Transcriptional start points. To corroborate the proper operation of the promoter fragments analyzed and to identify the promoter(s) responsible for *gfp* expression in each case, primer extension analysis was carried out with primers corresponding to the *gfp* gene and RNA isolated from the CSEL strains grown with ammonium and incubated in the absence of combined N. Similar results were obtained with primers GFP4 (Fig. 3) and GFP3 (not shown). The three transcripts that are generated by the native *ntcA* promoter region, which correspond to the TSPs −49, −136, and −180 (21), were observed by using RNA isolated from filaments of the control strain CSEL6 incubated in the absence of combined N. Whereas the P_2 promoter (rendering TSP −136) was utilized constitutively, P_1 (rendering TSP −49) and P_3 (rendering TSP −180) were induced after N step-down. This result indicates that the *ntcA* promoter cloned in the heterologous *nuiA-nucA* location works properly and, thus, contains all the elements necessary for the regulated expression of the three transcripts.

Consistent with the introduced deletions, the transcript corresponding to the −180 TSP was not observed using RNA from strain CSEL7, and neither the −180 nor the −136 transcript was observed using RNA from strain CSEL8 or CSEL9 (Fig. 3). The −49 transcript was observed using RNA from the four strains (CSEL6 through CSEL9). Noteworthy was the observation that the utilization of the TSP at −49 was higher in strain CSEL8 than in any of the others, even in ammonium-grown filaments. The utilization of the −49 TSP increased in strain CSEL9 upon N step-down. The increases in *gfp* transcript levels in strains CSEL8 and CSEL9 were confirmed by Northern blot analysis (not shown).

Concluding remarks. Using an *ntcA-gfp* translational fusion, we have shown that the induction of the *ntcA* gene that takes place after an N step-down in *Anabaena* sp. strain PCC 7120 corresponds, to a significant extent, to increased expression in cells that are differentiating into a heterocyst. Localized induction of the *ntcA-gfp* fusion takes place promoted by a 603-bp fragment from the DNA immediately upstream of *ntcA* (Fig. 2, strain CSEL6). The removal of DNA upstream from position −182 hampered this induction, suggesting an important role of the P_3 promoter (rendering the −180 TSP) in the localized expression of *ntcA*. Previous results by Ramasubramanian et al. (24) also indicated that the DNA region corresponding to this promoter is essential for diazotrophic growth. Although transient induction of P_3 in filaments subjected to N step-down is NtcA and HetR dependent (21), no NtcA binding site in the P_3 promoter can be identified. NtcA-dependent promoters that do not have recognizable NtcA-binding sites and may therefore be indirectly regulated by NtcA have also been described for the *hetR* (21) and *glnA* genes (27) of *Anabaena* sp. strain PCC 7120.

Further removal of DNA upstream from position −143 results in derepressed expression of *gfp* in ammonium-grown filaments, although an induced and localized expression is still observed after N step-down (Fig. 2, strain CSEL8). Derepression observed for strain CSEL8 indicates that the DNA fragment from −143 to −182 has a negative effect on expression, which could be mediated by the binding of a repressor. Our results also show that P_1 is the only *ntcA* promoter used in strain CSEL8 (Fig. 3). Because the induction of P_1 is HetR and NtcA dependent (21), and because two putative NtcA binding sites (centered at −143.5 and −103.5 with respect to the translational start of the gene, respectively) are present upstream of position −49, direct activation of transcription by NtcA may represent a molecular mechanism for the localized induction of P_1. The deletion of DNA covering both binding sites abolishes localized induction (Fig. 2, strain CSEL9), whereas the deletion of DNA covering only half of the −143.5 site does not (Fig. 2, strain CSEL8). The −143.5 NtcA binding site is centered at 94.5 and the −103.5 site at 54.5 nucleotides upstream of the TSP instead of the usual location in standard class II NtcA-dependent promoters, which is about 41.5 nucleotides upstream from the TSP (2, 10). It is possible that NtcA activates the P_1 promoter by binding to these two binding sites. Because the binding sites are 40 nucleotides apart from each other, NtcA dimers would bind in the same face of the double helix, facilitating a synergistic mechanism of transcription activation. On the other hand, transcription from the −49 TSP and its nonlocalized induction still occurs in strain CSEL9 (Fig. 2 and 3). The mechanism of operation of P_1 in the absence of any NtcA binding site is currently unknown.

In summary, in contrast to P_2, which is constitutively utilized in the *Anabaena* filaments, P_1 and P_3 appear capable of inde-

pendently driving the induction of the *ntcA* gene in developing cells. Localized induction of *ntcA* in developing heterocysts is consistent with an important role of the NtcA transcription factor beyond the initiation of heterocyst differentiation (11).

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