All4312, an NtcA-regulated two-component response regulator in *Anabaena* sp. strain PCC 7120

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**Abstract**

All4312, encoded by open reading frame all4312 in the genome of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120, exhibits a CheY-like receiver domain and an output domain similar to that of OmpR, characteristic of two-component response regulators. Expression of all4312 was directly regulated by NtcA, the global transcriptional regulator of nitrogen assimilation in cyanobacteria. Features characteristic of NtcA-activated promoters were also found upstream from genes encoding All4312 homologues in several other cyanobacterial genomes. Expression of all4312 was however unaffected in a mutant of hetR, which encodes a regulator triggering heterocyst development. The function of All4312 may be related to the cellular response to nitrogen deprivation.

**Introduction**

Cyanobacteria are a group of widely distributed phototrophic prokaryotes that carry out oxygenic, plant-type photosynthesis. Cyanobacteria are able to use different nitrogen sources including nitrate and ammonium and many strains can also fix atmospheric nitrogen. Ammonium is assimilated in preference over nitrate, which is used in preference over dinitrogen (Flores & Herrero, 1994; Herrero *et al*., 2001). Some filamentous cyanobacteria, including *Anabaena* spp., are able to differentiate, in response to nitrogen deficiency, cells specialized in nitrogen fixation called heterocysts. Assimilation of different nitrogen sources is globally regulated in these organisms by NtcA, a transcriptional regulator belonging to the CAP (or CRP) family that, in the absence of ammonium, activates the expression of genes required for the assimilation of alternative nitrogen sources including atmospheric nitrogen (Vega-Palas *et al*., 1992; Frías *et al*., 1994; Luque *et al*., 1994; Wei *et al*., 1994; Herrero *et al*., 2001). NtcA binds to specific sites in the promoter regions of the regulated genes and activates their expression in response to ammonium withdrawal (Luque *et al*., 1994). The structure of consensus NtcA-binding sites has been defined (Luque *et al*., 1994) and several NtcA-activated promoters have been shown to carry an NtcA-binding sequence in the form GTAN₈TAC, which is located about 22 nucleotides upstream from the promoter – 10 hexamer (Herrero *et al*., 2001). NtcA-binding sites with a repressor, rather than activating, role have been identified in a few cases (Herrero *et al*., 2001). The NtcA protein appears to have as a positive effector 2-oxoglutarate (Vázquez-Bermúdez *et al*., 2002, 2003; Luque *et al*., 2004), which is an indicator of the C to N balance in cyanobacterial cells (Muro-Pastor *et al*., 2001). For some promoters, the PII protein is also needed for full activation by NtcA under N deficiency (Aldehni *et al*., 2003; Paz-Yepes *et al*., 2003).

A number of cases have been described in which NtcA-mediated nitrogen regulation is not directly operated by NtcA (Herrero *et al*., 2001, 2004). In those cases, one would expect that NtcA activates the expression of regulatory proteins that would then be responsible for direct regulation, but no such effector is yet known. Here we describe a protein with homology to two-component response regulators whose expression is directly operated by NtcA in *Anabaena* sp. strain PCC 7120.

**Materials and methods**

**Strains and growth conditions**

This study was carried out with the heterocyst-forming cyanobacterium *Anabaena* sp. (also known as *Nostoc* sp.)
strain PCC 7120 and derivative strains CSE2, an insertional mutant of the ntcA gene (Frias et al., 1994), and 216, which bears a point mutation in the hetR gene (Buikema & Haselkorn, 1991). They were grown photautotrophically at 30 °C in BG110C medium [BG11 medium (Rippka et al., 1979) without NaN3, supplemented with 10 mM of NaHCO3] supplemented with 6 mM NH4Cl plus 12 mM N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES)–NaOH buffer (pH 7.5), bubbled with a mixture of CO2 and air [1% volume in volume (v/v)], and supplemented with 2 μg mL−1 of streptomycin and 2 μg mL−1 of spectinomycin in the case of strain CSE2 and those strains bearing fusions between the region upstream from all4312 and the gfp gene.

For RNA isolation, cells growing exponentially in BG110C medium supplemented with NH4Cl were harvested at room temperature and either used directly (time 0) or washed with BG110C medium, resuspended in BG11C (nitrate-containing) or in BG11C (nitrogen-free) medium and further incubated under culture conditions for the number of hours indicated in each experiment.

For the analysis of gfp expression, cells growing exponentially in BG110C medium supplemented with NH4Cl were harvested at room temperature and either used directly (time 0) or washed with BG110C medium, resuspended in BG11C (nitrate-containing) or in BG11C (nitrogen-free) medium and further incubated under culture conditions for the number of hours indicated.

Escherichia coli strains were grown in Luria broth (LB) supplemented, when necessary, with antibiotics added at standard concentrations (Ausubel et al., 2005).

DNA and RNA isolation and manipulation

Total RNA from Anabaena sp. strain PCC 7120 and its derivatives was isolated as previously described (Muro-Pastor et al., 2002). Primer extension analysis of the all4312 transcripts was carried out as described previously (Muro-Pastor et al., 1999). The oligonucleotide used as primer was all4312-1 (complementary to positions +104 to +84 relative to the translation start of all4312). Plasmid pCSAM113 (see below) was used to generate dideoxy-sequencing ladders using the same primer. Sequencing was carried out by the dideoxy chain-termination method, using a T7SequencingTM kit (Amersham Biosciences, Uppsala, Sweden) and [γ-32P]-thio dATP. Northern analysis was carried out as described (Muro-Pastor et al., 1999). The all4312 probe used was a 714-bp Ncol–HincII internal fragment that covers almost the whole open reading frame, isolated from pCSAM115.

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 (Ausubel et al., 2005).

Plasmids

Plasmid pCSAM113 contains a 602 bp DNA fragment PCR-amplified using oligonucleotides all4312-2 (corresponding to positions −488 to −467 with respect to the translational start of all4312) and all4312-1 (see above) and chromosomal DNA from Anabaena sp. strain PCC 7120 as template, cloned into the pGEM-T vector (Promega, Madison, WI). In pCSAM113a the orientation of the insert is such that sequences corresponding to the all4312-1 oligonucleotide are close to the SpeI site in the polynucleotide pGEM-T. In pCSAM113b the insert is cloned in the opposite orientation. Plasmid pCSAM115 contains a 1102 bp DNA fragment PCR-amplified with oligonucleotides all4312-Nco (corresponding to positions −12 to +10 with respect to the translational start of all4312, introducing a Ncol site at the start codon) and all4312-3 (complementary to positions +331 to +309 with respect to the translational stop of all4312) cloned into the pGEM-T vector. This fragment contains the complete all4312 ORF plus 331 bp downstream of all4312.

Disruption of all4312

all4312 in plasmid pCSAM115 was disrupted by introducing AccI-ended SmSp-resistance cassette C.S3, excised from pRL463 [pRL138/LHEH1(BamHI)/C.S3, nomenclature as in Elhai & Wolk, 1988a], into the ClaI site internal to all4312 rendering pCSAM116. The ca. 3.5-kbp PvuII fragment from pCSAM116, containing the disrupted all4312 plus some downstream sequences (all4312::C.S3), was cloned into the Klenow-filled BglII site of sacB vector pRL278 (Black et al., 1993). The resulting plasmid, pCSAM118 was transferred to Anabaena sp. strain PCC 7120 by conjugation as described (Elhai & Wolk, 1988b), using the helper plasmid pRL623 (Elhai et al., 1997), and SmSp-resistant colonies were selected. Isolation of double recombinants was attempted but, under our culture conditions, no sucrose-resistant, Nm-sensitive colony could be obtained in several rounds of selection.

Fusions to the green fluorescent protein

Fusions of the promoter region of all4312 to the gfp gene encoding green fluorescent protein were prepared as follows. SmSp-resistance cassette C.S3, excised from pRL463 (see above) as an XbaI fragment, was inserted into the SpeI site located in the polynucleotide of pCSAM113b upstream from the all4312 promoter, rendering pCSAM114. A SalI–Ncol (Klenow-filled) fragment from pCSAM114, containing C.S3 followed by the promoter region of all4312, was placed upstream from the gfp gene in SalI, EcoRV-digested pCSEL19, rendering pCSAM117 [pCSEL19 contains a promoterless gfp gene PCR-amplified using oligonucleotides...
gfp-1 (5’GGAGATATCATGACATGGG3’, introducing an EcoRV site upstream from the start codon of the gfp gene) and gfp-2 (5’AACAGAGTCCCTGACATGGG3’) and plasmid pKEN2-GFPmut2 (Ezzaw-Nikpay et al., 1994; Cormack et al., 1996) as a template, cloned into the pGEM-T vector in the same orientation of the β-lactamase gene. A PstI fragment from pCSAM117, containing C.S3 followed by a transcriptional fusion between the region upstream from all4312 and the gfp gene, was cloned in both orientations into the PstI site of pCSAV80 [a derivative of pCSAM28 (Muro-Pastor et al., 1992) in which the nucA gene has been inactivated by digestion with HindIII followed by Klenow treatment and religation], designed for integration of constructs into the nucA region located in the α-megaplasmid of Anabaena sp. strain PCC 7120. The resulting plasmids, pCSAM119a and pCSAM119b, were transferred to Anabaena by conjugation as described above and SmSp resistant colonies were selected. The C.S3 cassette bears transcriptional terminators that are effective in Anabaena sp. strain PCC 7120 (Frias et al., 1997), ensuring that the Pall4312:gfp fusion is not transcribed from an external promoter other than Pall4312. The accumulation of GFP reporter was analysed by laser confocal microscopy. Samples were observed using a Leica HCX PLAN-APO 63X 1.4 NA oil immersion objective (Leica, Wetzlar, Germany). GFP was imaged using the 488 nm line supplied by an argon ion laser. Fluorescent emission was monitored by collection across windows of 500–570 nm (GFP imaging) and 630–700 nm (cyanobacterial emission was monitored by collection across windows of 660–700 nm). All confocal images were collected using the same settings, so that the intensities can be compared.

**Band-shift assays**

A 330 bp DraI–SpeI fragment from pCSAM113a was used in band shift assays with purified NtcA. This fragment includes sequences –220 to +104 with respect to the translation start of all4312. DNA fragments were end-labelled with T4 polynucleotide kinase and [γ-32P]dATP. Assays were carried out as described previously (Luque et al., 1994) in the presence or absence of 0.6 mM 2-oxoglutarate (Vázquez-Bermúdez et al., 2002), and they contained about 0.5 fmol of labelled fragment and 2.5–15 pmol of purified His-tagged NtcA (Muro-Pastor et al., 1999).

**Results**

**Expression of all4312**

As a result of a search for NtcA boxes upstream from Anabaena sp. strain PCC 7120 ORFs and their corresponding homologues in Nostoc punctiforme, all4312 was identified as a gene exhibiting a putative regulatory NtcA box in similar positions in both organisms (J. Elhai and A. M. Muro-Pastor, unpubl. results). Genes putatively encoding homologues of All4312 were identified in 10 cyanobacterial genomes. The regions upstream from the corresponding open reading frames contained NtcA boxes with the consensus sequence GTAN8TAC centered at positions ranging from 66 to 130 nucleotides upstream of the predicted translational start (Fig. 1). Furthermore, in four cases, the NtcA box contains the nucleotides CA in the second and third positions after the GTA triplet, a feature conserved in many consensus-type NtcA-binding sites (Herrero et al., 2001). The observation that NtcA boxes are located in such a position in all ten genomes suggests that NtcA might be involved in expression of the corresponding genes.

Because NtcA is known to regulate expression of genes in response to the nitrogen status of the cells, expression of all4312 in Anabaena sp. strain PCC 7120 was analysed in ammonium-grown filaments incubated for 4 or 24 h in the presence of nitrate or in the absence of combined nitrogen.

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**Fig. 1.** Identification of putative NtcA boxes in the regions upstream from genes encoding All4312 and its homologues in several cyanobacterial genomes. Sequences are aligned at the predicted NtcA boxes and distances to the putative translation start (GTC or ATG codon) of the corresponding genes are indicated. The transcription start point determined for all4312 is underlined, and the corresponding putative promoter − 10 hexamer is indicated. Sequences aligned correspond to those upstream from all4312 (first line) and genes encoding All4312 homologues from Anabaena variabilis strain ATCC 29413 (gi75907486), Nostoc punctiforme strain PCC 73102 (gi53687472), Trichodesmium erythraeum strain IMS101 (gi71676548), Synechocystis sp. strain PCC 6803 (gi16332107), Crocosphaera watsonii strain WH 8501 (gi67924898), Synechococcus elongatus strain PCC 7942 (gi45513869), Synechococcus elongatus strain PCC 6301 (gi56751647), Gloeobacter violaceus strain PCC 7421 (gi35212842), and Thermosynechococcus elongatus strain BP-1 (gi22295054).

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also analysed for comparison. Several Anabaena clones were analysed by northern hybridization (Fig. 2a) and primer extension (Fig. 3). Expression of the all4312 promoter region in the mutant strain CSE2 was similar to that observed in the wild type. Northern blot hybridization (Fig. 2a) showed that expression in the wild-type strain was very low in the presence of ammonium, was slightly induced in the presence of nitrate as sole nitrogen source and was strongly induced after 4 h of nitrogen deficiency. A time-course of induction of all4312 in response to nitrogen deprivation was also carried out (Fig. 2b). Induction of all4312 took place, in wild-type cells, after less than 3 h of nitrogen deficiency, and expression was highest between 6 and, at least, 12 h. Induction of expression did not take place in the ntcA mutant strain CSE2. Transcript 5′ ends for the all4312 gene in Anabaena sp. strain PCC 7120 were analysed by primer extension (Fig. 3). A unique transcription start point was identified 27 nucleotides upstream from the translation start of all4312. Consistent with northern hybridization results shown in Fig. 2, expression from this start point was very low in ammonium-grown cells and increased after 3 h of nitrogen deficiency. Also, the 5′ end of this mRNA could not be detected in the ntcA mutant CSE2 (Fig. 3).

Expression of all4312 in the hetR mutant strain 216 was also analysed by northern hybridization (Fig. 2b) and primer extension (Fig. 3). Expression of all4312 was not altered in the hetR mutant, and utilization of the transcription start point located 27 nucleotides upstream from the translational start of all4312 in the hetR mutant was similar to that observed in the wild type.

**Analysis of gfp fusions**

Expression of transcriptional fusions of the all4312 promoter to a promoterless gfp gene was analysed in ammonium-grown filaments incubated in nitrogen-free medium for 9 or 24 h. Ammonium-grown filaments were also analysed for comparison. Several Anabaena clones carrying fusions to gfp were analysed, and the results for two clones, bearing the promoter-gfp fusion in both orientations with respect to vector sequences, are shown in Fig. 4. GFP fluorescence of strains carrying the Pall4312::gfp fusions was low in ammonium-grown filaments but was higher after nitrogen deprivation. Expression of transcriptional fusions was similar in all cells of the filament. Some highly fluorescent cells appeared occasionally, but no correlation could be established with proheterocysts or mature heterocysts.

**Analysis of the all4312 promoter**

The analysis of the region upstream from all4312 indicated that the transcription start point determined by primer extension was located at the standard distance from the NtcA boxes found in consensus Class II NtcA-activated promoters. Thus, the NtcA box is centered at about — 41.5 nucleotides with respect to the tsp, which is preceded by a — 10 box in the form TAN₅T (see Fig. 1). Band-shift assays were carried out with purified NtcA protein and a fragment from the all4312 upstream region containing the NtcA box (Fig. 5). Retardation of the labelled DNA fragment in response to the addition of increasing amounts of NtcA was observed, indicating binding of NtcA. Affinity of NtcA for this promoter fragment was higher in the presence of 2-oxoglutarate.

**Discussion**

The observations described in this work indicate that expression of all4312 is directly regulated by NtcA, the global transcriptional regulator for N control in cyanobacteria. In response to nitrogen deficiency, an increase in the expression of the ntcA gene (Muro-Pastor et al., 2002) and
accumulation of the NtcA protein (Olmedo-Verd et al., 2005) take place in Anabaena sp. strain PCC 7120. Such increased expression of NtcA requires HetR, a positive-acting factor for heterocyst differentiation (Buikema & Haselkorn, 1991). Because HetR is required for the transient increase of NtcA levels that takes place during nitrogen deficiency (Muro-Pastor et al., 2002), the observation that HetR is not involved in the expression of all4312 suggests that those increased levels of NtcA are not required for all4312 expression. Expression of all4312 would thus result from the activation of NtcA in response to nitrogen deficiency. Combined nitrogen deprivation would provoke an increase in the cellular levels of 2-oxoglutarate (Laurent et al., 2005), which has the effect of increasing NtcA affinity for binding to the all4312 promoter (Fig. 5). Thus, one would predict that this promoter is activated early, and probably in all cells of the filament, upon combined nitrogen deprivation (Herrero et al., 2004). This appears to be actually the case, as shown by the Pull4312-gfp transcriptional fusions analysed in this work (Fig. 4). A number of promoters are activated by NtcA upon ammonium withdrawal (Herrero et al., 2001). One of them, the glnA P1 promoter (Valladares et al., 2004), bears an NtcA binding site that is identical in sequence to that of the all4312 promoter characterized in this work.
Function related to N stress would also be consistent with the presence of All4312 homologues exhibiting similar regulatory features (i.e. NtcA boxes in their promoter regions) in non-nitrogen-fixing cyanobacteria.

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