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The NtcA-Dependent P_1 Promoter Is Utilized for *glnA* Expression in N_2-Fixing Heterocysts of *Anabaena* sp. Strain PCC 7120

Ana Valladares, Alicia M. Muro-Pastor, Antonia Herrero, and Enrique Flores*

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas-Universidad de Sevilla, Seville, Spain

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Expression of the *glnA* gene encoding glutamine synthetase, a key enzyme in nitrogen metabolism, is subject to a variety of regulatory mechanisms in different organisms. In the filamentous, N_2-fixing cyanobacterium *Anabaena* sp. strain PCC 7120, *glnA* is expressed from multiple promoters that generate several transcripts whose abundance is influenced by NtcA, the transcription factor exerting global nitrogen control in cyanobacteria. Whereas RNA_I originates from a canonical NtcA-dependent promoter (P_1) and RNA_{II} originates from a σ^{70}-type promoter (P_2), RNA_{IV} is influenced by NtcA but the corresponding promoter (P_3) does not have the structure of NtcA-activated promoters. Using RNA isolated from *Anabaena* filaments grown under different nitrogen regimens, we observed, in addition to these transcripts, RNA_V, which has previously been detected only in in vitro transcription assays and should originate from P_4. However, in heterocysts, which are differentiated cells specialized in N_2 fixation, RNA_I was the almost exclusive *glnA* transcript. Analysis of P_{glnA}::*lacZ* fusions containing different fragments of the *glnA* upstream region confirmed that fragments carrying P_1, P_2, or P_3 and P_4 have the ability to promote transcription. Mutation of the NtcA-binding site in P_1 eliminated P_1-directed transcription and allowed increased use of P_2. The NtcA-binding site in the P_1 promoter and binding of NtcA to this site appear to be key factors in determining *glnA* gene expression in vegetative cells and heterocysts.

Glutamine synthetase is a key enzyme in nitrogen metabolism in all living cells and constitutes the route for incorporation of inorganic nitrogen (in the form of ammonium) into organic material in many microorganisms and plants. The *glnA* gene encoding glutamine synthetase is subject to regulation and is expressed at maximal levels when cells are incubated under nitrogen-limiting conditions. Whereas activation of expression of *glnA* in enteric bacteria is mediated by the well-characterized NtrB-NtrC two-component regulatory system (27), in cyanobacteria transcription of the *glnA* gene is under control of NtcA, the global nitrogen control transcription factor of these organisms (19). The glutamine synthetase protein is abundant in cyanobacteria (26), and *glnA* is a predicted highly expressed gene (30) whose transcript is readily detected (15, 45).

The cyanobacteria, which belong to the domain *Bacteria*, are characterized by performing oxygenic photosynthesis and are considered the evolutionary precursors of algal and higher-plant chloroplasts (17). The cyanobacterial RNA polymerase is similar to the well-characterized enterobacterial enzyme (43), although the *rpoC* gene encoding the RNA polymerase β′ subunit is split in the cyanobacteria into two genes, *rpoC1* and *rpoC2*, which encode polypeptides γ and β′, which are homologous to the N-terminal and C-terminal halves of enterobacterial β′, respectively (3, 53). Thus, the architecture of the cyanobacterial RNA polymerase core is $\alpha_2\beta\beta'\gamma$ instead of the enterobacterial $\alpha_2\beta\beta'$ (43). The principal RNA polymerase σ factor in *Anabaena* sp. strain PCC 7120 (the product of the *sigA* gene) is homologous to the vegetative *Escherichia coli* σ^{70} and *Bacillus subtilis* σ^{43} factors (5). Consistently, a number of cyanobacterial gene promoters have been found to bear a −10 box in the form TAN_3T (9, 22), and some of them also carry a recognizable −35 box (42).

Although numerous cyanobacterial promoters do not contain an obvious −35 box, some have an extended −10 region ($TGNTAN_3T$) similar to that found in a subclass of *E. coli* promoters that function without a recognizable −35 box (2, 10). This is the case for the promoter for the *rbcLXS* operon encoding ribulose-1,5-bisphosphate carboxylase/oxygenase. NtcA-dependent promoters represent another class of promoters that do not contain a recognizable −35 box. These promoters possess a −10 box (TAN_3T) and an NtcA-binding site characterized by the signature sequence $GTAN_8TAC$, which is usually centered at about position −41.5 with respect to the transcription start site (19). NtcA belongs to the CAP family of bacterial transcriptional regulators, and the promoter structure just described is similar to that of the class II CAP-dependent promoters (7). NtcA functions in response to the C/N balance of the cell, and 2-oxoglutarate has been identified as a putative effector of NtcA (35, 44, 46–48). The *ntcA* gene appears to be autoregulatory, and *ntcA* expression, which takes place at a basal level in the presence of ammonium, increases in response to nitrogen limitation (21, 22, 34, 38, 49).

Under combined nitrogen limitation conditions, filamentous cyanobacteria like *Anabaena* sp. strain PCC 7120 differentiate heterocysts, which are specialized cells that are the sites of N_2 fixation in aerobically grown filaments (13). Heterocyst differentiation does not take place in *ntcA* mutants (15, 49) or in mutants with mutations in the development regulatory gene *hetR* (4, 6). The extensive structural and physiological differ-

* Corresponding author. Mailing address: Instituto de Bioquímica Vegetal y Fotosíntesis, Centro de Investigaciones Científicas Isla de la Cartuja, Avda. Américo Vespucio s/n, E-41092 Seville, Spain. Phone: 34 95 448 9523. Fax: 34 95 446 0065. E-mail: eflores@ibvf.csic.es.

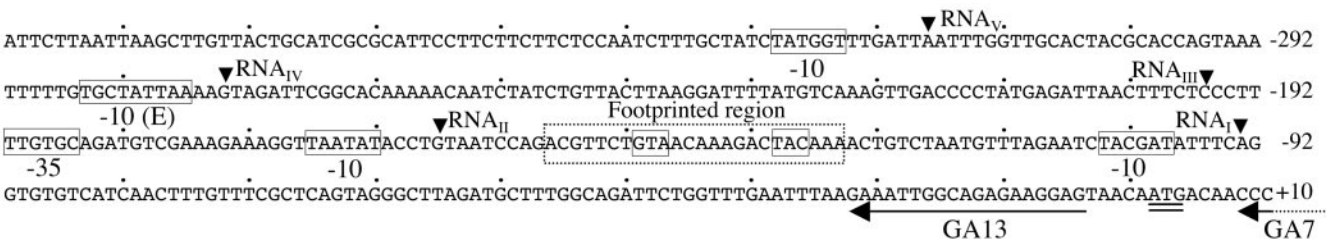

FIG. 1. Sequence of the *Anabaena glnA* genomic region from nucleotide 391 upstream of the translation start to nucleotide 10 downstream of the translation start. RNA 5′ ends identified in this work (which do not always exactly coincide with those previously reported) are indicated, along with the following possible promoter features: −10 boxes for RNA_I, RNA_{II}, and RNA_V, extended −10 box [−10 (E)] for RNA_{IV}, −35 box for RNA_{II}, and the P_1 NtcA-binding site. The NtcA-footprinted region (37), including the NtcA-binding site sequence signature ($GTAN_8TAC$), the *glnA* ATG start codon (double underlined), and the location of oligonucleotides GA7 and GA13 used for primer extension analysis are also indicated. The limits of the different DNA promoter fragments analyzed in this work were as follows: fragment C, positions 381 and 246; fragment CB, positions 381 and 139; fragment CBA, positions 381 and 70; fragment B, positions 237 and 139; fragment BA, positions 237 and 70; and fragment A, positions 159 and 70 (positions indicate nucleotides upstream from the *glnA* translation start).

ences between heterocysts and vegetative cells are largely the result of differential gene expression (50). Thus, for instance, *nifHDK* encoding the nitrogenase complex is expressed only in heterocysts, whereas *rbcLXS* is expressed only in vegetative cells (13). Some genes, however, have to be expressed in both types of cells. This is the case for *glnA* (13), since glutamine synthetase is involved in nitrogen assimilation in the vegetative cells and also provides the path for incorporation of the ammonium produced in the N_2 fixation reaction in the heterocysts (51).

The promoter region of the *glnA* gene in *Anabaena* sp. strain PCC 7120 has a complex structure and generates several different RNA species corresponding to RNA_I to RNA_V (Fig. 1). RNA_I, whose 5′ end is located 93 nucleotides upstream of the *glnA* translation start, is more abundant in the absence of ammonium than in the presence of ammonium (45), is NtcA dependent, and originates from a promoter (that we call P_1) with the canonical structure of the NtcA-dependent promoters (15, 19). Indeed, NtcA binds with high efficiency to its binding site in the P_1 promoter, and this NtcA-binding site has been footprinted (37). RNA_{II}, whose 5′ end is located 155 (45) or 157 (15) nucleotides upstream of the start codon, is detected independent of the nitrogen source, although its level is somewhat higher in the presence of ammonium than in the absence of ammonium. The promoter that originates RNA_{II}, P_2, is similar to the canonical σ^{70}-dependent promoters and can be used in an *E. coli* strain carrying the *Anabaena glnA* gene in a plasmid (45), as well as in in vitro transcription assays with both *Anabaena* and *E. coli* RNA polymerases (43). RNA_{III}, whose 5′ end has been located either 244 to 247 (45) or 196 (15) nucleotides upstream of the translation start, may arise from processing of RNA_{IV} rather than represent a true transcription start site (42). There is some uncertainty about the exact 5′ end of RNA_{IV}; it is 273 (43), 275 (15), or 266 (45) nucleotides upstream of the start codon. However, the region from nucleotide 282 to nucleotide 277 upstream of the translation start has a sequence that represents an acceptable σ^{70}-type −10 box (TATTAA). Interestingly, considering a few upstream nucleotides, a sequence (TGNTATTAA) that could represent an extended −10 box is evident. The promoter that generates RNA_{IV}, P_3, has been described as NtcA dependent (15), although it has no evident NtcA-binding site, and it can be used in in vitro transcription assays with both *Anabaena* and *E. coli* RNA polymerases (43). Finally, RNA_V, whose 5′ end is located 319 nucleotides upstream of the translation start, corresponds to a transcript that has been detected only in in vitro transcription assays (43), although the putative promoter for this transcription start point has a recognizable −10 box (42).

The complex pattern of expression summarized above has been interpreted in terms of expression of the *glnA* gene mainly from a σ^{70}-type promoter (P_2) in ammonium-grown *Anabaena* cells and from a *nif*-like promoter (P_1, which was later identified as an NtcA-type promoter [15]) under N_2-fixing conditions (45). In this work, we show that P_1 is the promoter used in heterocysts.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Anabaena* sp. strain PCC 7120 was grown axenically in BG11 medium (39), which contains 17.6 mM $NaNO_3$, in $BG11_0$ (nitrogen-free) medium, or in $BG11_0$ medium supplemented with 2 mM NH_4Cl and 4 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)–NaOH buffer (pH 7.5). For plates, the media were solidified with separately autoclaved 1% agar (Difco). Cultures were grown at 30°C in the light (75 microeinsteins $\cdot m^{-2} \cdot s^{-1}$) with shaking (80 to 90 rpm) for liquid cultures. *Anabaena* sp. strain PCC 7120 derivatives carrying a Km^r Nm^r gene cassette (12) were routinely grown in medium supplemented with 25 μg of neomycin $\cdot ml^{-1}$, and strains carrying Sm^r Sp^r gene cassette C.S3 (12, 36) were grown in medium supplemented with 2 to 5 μg of spectinomycin $\cdot ml^{-1}$ and 2 to 5 μg of streptomycin $\cdot ml^{-1}$. The chlorophyll *a* contents of the cultures were determined by using methanolic extracts of cells (23).

For RNA isolation, cells growing exponentially in $BG11_0C$ medium ($BG11_0$ medium plus 10 mM $NaHCO_3$) or $BG11_0C$ medium supplemented with 5 to 8 mM NH_4Cl (plus a double concentration of TES-NaOH buffer [pH 7.5]) and bubbled with a mixture of CO_2 and air (1:99, vol/vol) were used. For induction experiments, cells grown exponentially in $BG11_0C$ medium supplemented with 5 to 8 mM NH_4Cl were harvested at room temperature, washed with and resuspended in $BG11_0C$ medium, and then incubated under culture conditions for the numbers of hours indicated below. Cultures used for isolation of RNA from heterocysts were grown in $BG11_0C$ medium supplemented with 5 to 8 mM NH_4Cl until the chlorophyll *a* concentration reached 3 to 5 μg $\cdot ml^{-1}$. Cells were then washed with and resuspended in $BG11_0C$ medium and incubated until mature heterocysts were observed (19 h). Heterocysts were isolated as described previously (18).

E. coli strain DH5α was grown in Luria-Bertani medium containing, when necessary, 50 μg of ampicillin $\cdot ml^{-1}$, 50 μg of kanamycin $\cdot ml^{-1}$, 30 μg of chloramphenicol $\cdot ml^{-1}$, 25 μg of streptomycin $\cdot ml^{-1}$, or 100 μg of spectinomycin $\cdot ml^{-1}$.

β-Galactosidase activity. β-Galactosidase activity was determined as previously described (48) by using chloroform-treated filaments from shaken cultures

TABLE 1. Deoxyoligonucleotide primers used in this work

Primer	Sequence (5′-3′)
GA1	AAGCCTGTTACTGCATCGCGCATTCC
GA2	CAGATAGATTGTTTTTGTGCC
GA3	GGATTTTATGTCAAAGTTGACCCC
GA4	TACAGAACGTCTGGATTACAGG
GA5	CGAAAGAAAGGTTAATATTACCTG
GA6	CGAAACAAAGTTGATGAC
GA7	CTTTTCAAGACTTCTTGTGGGGG
GA9	CCAGACGTTCTCATACAAAGACTAC
GA10	GTAGTCTTTGTATGAGAACGTCTGG
GA13	CTCCTTCTCTGCCAATTTC
LZ3	CAATCACTGCTCAATGCCC

that were grown in $BG11_0$ medium containing NH_4^+ and incubated for 3 days in $BG11_0$ medium with air levels of CO_2. One unit of β-galactosidase activity corresponded to production of 1 μmol of *o*-nitrophenol · min^{-1}. The data presented below are the means and standard deviations for results obtained with three to six independent cultures. The protein concentration was determined by a modified Lowry procedure (24) by using 0.2-ml aliquots of the cultures.

Mutagenesis of the NtcA-binding site in the *glnA* P_1 promoter. The deoxyoligonucleotide primers used in this work are shown in Table 1. Site-directed mutagenesis of the P_1 promoter NtcA-binding site was carried out by PCR as described previously (1). The mutagenic oligonucleotides used were GA9 and GA10, and the flanking oligonucleotides were GA1 and GA7; pAN503, which contains the upstream region and coding sequence of the *glnA* gene (14), was used as the template. After cloning of the PCR-generated fragments, the insert of the plasmid generated, pCSAV117, containing the mutated version (CBA*) of the *glnA* upstream region, was sequenced by using oligonucleotide GA6 in order to check that it contained only the desired change (CAT instead of GTA).

Construction of *Anabaena* strains with transcriptional fusions. Different fragments from the *glnA* gene upstream region were amplified by PCR by using plasmid pAN503 as the template and oligonucleotides GA1 plus GA2, GA1 plus GA4, GA1 plus GA6, GA3 plus GA4, GA3 plus GA6, and GA5 plus GA6, which resulted in fragments C, CB, CBA, B, BA, and A, respectively (see Fig. 3). The amplified products were cloned in vector pGEM-T (Promega). SphI/SalI fragments from the resulting plasmids (containing the cloned fragment) and from plasmid pCSAV117 (which bears fragment CBA*) were inserted between SphI and SalI sites in vector pIC20R (25). A 2-kb Sp^r Sm^r gene cassette with HindIII ends was inserted into the unique HindIII site of pIC20R. A BamHI fragment from pPE20 containing the promoterless *lacZ* gene (20) was cloned into a BamHI site just after the *glnA* promoter fragment. A plasmid with no promoter was also constructed by using the same steps and was used as a negative control. In order to integrate these constructs bearing transcriptional fusions into the cyanobacterial genome, they were transferred to plasmid pCSAV81. This plasmid consists of pCSAM28, which contains a fragment of the *nucA* region from *Anabaena* sp. strain PCC 7120 cloned between the EcoRI and EcoRV sites of pBR322 (31), in which the *nucA* gene was mutated by digestion with HindIII and filling in with the Klenow enzyme (this inactivation was necessary because the increase in *nucA* expression directed from the Km^r cassette inserted upstream of this gene could be lethal for the *E. coli* host). BglII fragments containing the Sm^r Sp^r cassette, the promoter fragment, and the *lacZ* gene were ligated to BglII-digested pCSAV81, generating plasmids pCSAV44, pCSAV45, pCSAV46, pCSAV47, pCSAV48, pCSAV49, and pCSAV127 containing promoter fragments C, CB, CBA, B, BA, A, and CBA*, respectively, and pCSAV50 containing no promoter fragment.

In vitro-generated constructs carrying transcriptional fusions between different fragments of the *glnA* upstream region and the *lacZ* gene were transferred by conjugation (52) to *Anabaena* sp. strain PCC 7120 and, as indicated below, also to strain CSE2 (*ntcA* [15]) to generate strains bearing these transcriptional fusions in the *nucA* genomic region. For generation of these strains, *E. coli* HB101 containing plasmid pCSAV44, pCSAV45, pCSAV46, pCSAV47, pCSAV48, pCSAV49, pCSAV50, or pCSAV127 and helper plasmids pRL528 and pRL591-W45 (11) was mixed with *E. coli* ED8654 carrying the conjugative plasmid pRL443 and then with *Anabaena* sp. Exconjugants were isolated and identified as clones resistant to neomycin, streptomycin, and spectinomycin, and their chromosome structure in the *nucA* region was confirmed by Southern analysis.

DNA and RNA isolation and analysis. Total DNA (8) and RNA (34) from *Anabaena* sp. strain PCC 7120 and its derivatives were isolated as previously described. Sequencing was carried out by the dideoxy chain termination method by using a T^7Sequencing kit (Amersham Biosciences) and [α-^{35}S]thio-dATP. DNA fragments were purified from agarose gels with a Geneclean II kit (Bio 101). 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, 41).

Southern analysis was carried out by standard methods by using Hybond-N^+ membranes (Amersham Biosciences). 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 Biosciences) were carried out by using 0.1 M NaOH. Hybridization was performed at 65°C according to the recommendations of the manufacturers of the membranes. The *nifH* and *rbcL* probes were internal fragments of these genes amplified by PCR. All probes were ^{32}P labeled with a Ready to Go DNA labeling kit (Amersham Biosciences) by using [α-^{32}P]dCTP.

Primer extension analysis was carried out as described previously (33). The oligonucleotides used for analysis of the *glnA* transcript were GA7 and GA13. Plasmid pAN503 was used to generate dideoxy sequencing ladders by using the same primers. For determination of transcription start points of P_{glnA}::*lacZ* fusions, oligonucleotide LZ3 (complementary to a sequence located upstream of the *lacZ* gene) was used as the primer, and plasmid pCSAV127 was used to generate dideoxy sequencing ladders by using the same primer. Images of radioactive filters were obtained and quantified by using a Cyclone storage phosphor system and the OptiQuant image analysis software (Packard). Primer extension analyses were carried out with five (primer extensions with the GA7 and GA13 primers) or four (primer extensions with the *lacZ* primer) independent RNA preparations or with two independent preparations in the case of heterocyst RNA, and a representative example is shown in each case below.

Band shift assays and DNase I footprinting. DNA fragments to be used in electrophoretic mobility shift assays were obtained by PCR amplification. Oligonucleotides GA1, GA2, GA3, GA4, GA5, and GA6 (Table 1; see Fig. 3A) and plasmids pAN503 and pCSAV117 were used for PCR amplification of the *glnA* upstream region (wild-type and mutated versions, respectively). Oligonucleotides (pUC/M13 forward and reverse primers) and plasmid pBluescript SK(+) were used for amplification of a DNA fragment that was used as a negative control. Binding assays were carried out as described previously (28) by using as a source of NtcA a cell extract (1.4 μg of protein) of *E. coli* strain BL21(pCSAM70, pREP4), which overproduces the *Anabaena* sp. strain PCC 7120 NtcA protein (33), and, as a control, a cell extract of *E. coli* strain BL21(pQE9, pREP4).

The DNase I protection assay was carried out as described previously (16). The DNA used was the insert of pCSAV26 (containing fragment CBA in the pIC20R vector) limited by BamHI-EcoRV or HindIII-SacI sites and 3′ end labeled with the DNA polymerase Klenow fragment and [α-^{32}P]dCTP (3,000 Ci · $mmol^{-1}$). A cell extract of the *Anabaena* NtcA-overexpressing *E. coli* strain BL21(pCSAM70, pREP4) was used as a source of NtcA (33).

RESULTS

Transcription start points. To investigate the putative transcription start point(s) used for the *glnA* gene in heterocysts of *Anabaena* sp. strain PCC 7120, total RNA was extracted from heterocysts isolated from N_2-fixing filaments. This RNA preparation did not show any detectable hybridization with an *rbcL* probe, whereas it exhibited strong hybridization with a *nifH* probe, indicating that it consisted of highly specific heterocyst RNA (data not shown). Primer extension analysis with the GA7 (data not shown) or GA13 (Fig. 2A) primer was performed with the heterocyst RNA preparation and, as controls, with RNA preparations from whole filaments grown with ammonium or with N_2 as the nitrogen source. Consistent with previously reported data (15, 45), we observed several 5′ ends of *glnA* transcripts when RNA isolated from whole filaments was used. Transcription start points located 93 (corresponding to RNA_I) and 274 (RNA_{IV}) nucleotides upstream of the *glnA* start codon were used at somewhat higher levels in diazotrophically grown filaments than in ammonium-grown filaments, whereas the transcription start point located 157 nucle-

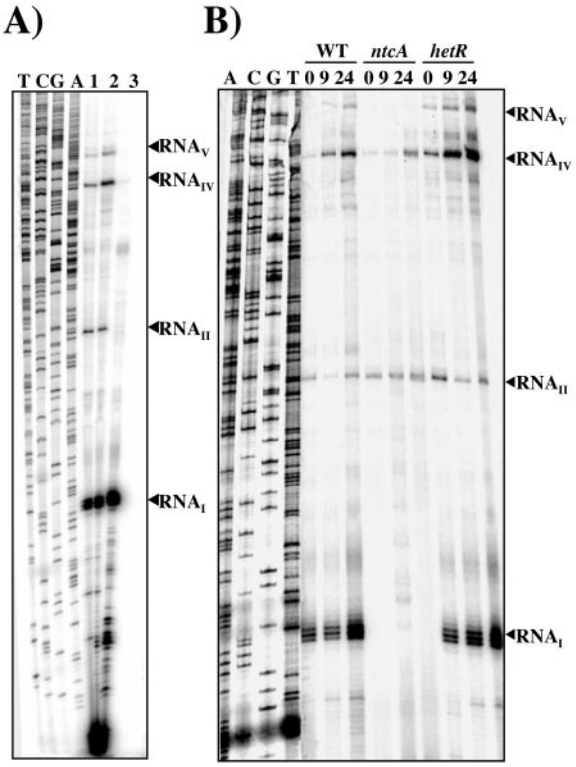

FIG. 2. Transcription start points for the *Anabaena glnA* gene. Primer extension analysis was carried out with primer GA13, and a sequencing ladder was generated with the *glnA*-containing plasmid pAN503 and the same primer. (A) RNA isolated from ammonium-grown filaments (lane 1), from N_2-grown filaments (lane 2), and from isolated heterocysts (lane 3) of *Anabaena* sp. strain PCC 7120. (B) RNA isolated from filaments of wild-type strain PCC 7120 (WT), *ntcA* strain CSE2 (*ntcA*), and *hetR* strain DR884a (*hetR*) grown with ammonium (lanes 0) or grown with ammonium and incubated for 9 h (lanes 9) or 24 h (lanes 24) without combined nitrogen. The arrowheads indicate the identified RNA 5′ ends.

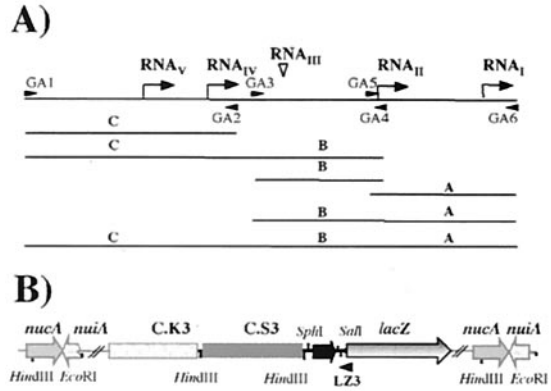

FIG. 3. Structure of P_{glnA}::*lacZ* fusions. (A) Schematic representation of the region from nucleotide 381 to nucleotide 70 upstream of the *Anabaena glnA* gene. The locations of the transcription start points generating RNA_I, RNA_{II}, RNA_{IV}, and RNA_V, the site defining RNA_{III}, and the oligonucleotides used for PCR amplification of the fragments shown below are indicated. (B) Structure of the *nucA* genomic region in the strains carrying P_{glnA}::*lacZ* fusions. The different promoter fragments shown in panel A (represented by a solid arrow) were cloned juxtaposed to C.S3 and *lacZ* to direct expression of *lacZ*. The approximate location of the LZ3 oligonucleotide primer is indicated. Note that an approximately 2.2-kb fragment of the *nucA* region is duplicated in these strains.

otides upstream of the start codon (corresponding to RNA_{II}) was used at similar levels in ammonium- and N_2-grown cells (Fig. 2A). The previously described RNA_{III}, considered to be a degradation product of RNA_{IV}, was observed only in some experiments and is not evident in Fig. 2. However, we observed a transcription start point 318 nucleotides upstream of the translation start that corresponded to RNA_V, a transcript previously detected only in in vitro transcription assays (43). RNA_V showed a regulatory pattern similar to that of RNA_{IV}. In contrast to these results obtained with RNA isolated from whole filaments, the heterocyst RNA preparation produced a strong signal that corresponded to RNA_I and only a faint signal corresponding to RNA_{IV}.

To test the effect of a *hetR* mutation on the use of the *glnA* transcription start points, RNA isolated from filaments grown with ammonium or grown with ammonium and subjected to nitrogen deprivation for several hours was used in primer extension assays with the GA7 (data not shown) or GA13 (Fig. 2B) primer. RNA preparations from *Anabaena* sp. strain DR884a (*hetR* [4]) and, as controls, from strain CSE2 (*ntcA* [15]) and wild-type strain PCC 7120 were used. Whereas in the *ntcA* mutant RNA_I was not detectable and RNA_{IV} was induced at a low level (15), both RNAs were expressed in the *hetR* mutant at levels similar to those found in the wild type (Fig. 2B). RNA_V was also NtcA dependent but independent of HetR (Fig. 2B).

Transcriptional fusions. To further analyze the promoter of the *glnA* gene, different DNA fragments of the *glnA* upstream region were cloned in front of a promoterless *lacZ* gene, and the resulting constructs were transferred to *Anabaena* sp. strain PCC 7120. A schematic representation of the DNA fragments used is shown in Fig. 3A. Each fragment was amplified by PCR and cloned juxtaposed to the C.S3 gene cassette, which bears transcription terminators, and the *lacZ* gene (Fig. 3B), and clones with the promoter fragment oriented so that it directed expression of *lacZ* were chosen (see Materials and Methods for details). These constructs were incorporated into the *nucA* region of the *Anabaena* genome, which is located in the α megaplasmid (32). This region was chosen because the *nucA* gene encodes a nuclease that is dispensable for growth of this cyanobacterium (31). The fusions were incorporated into *Anabaena* sp. strain PCC 7120 as described in Materials and Methods, which produced the genomic structure shown in Fig. 3B. To ensure that a correct clone, carrying a P_{glnA}::*lacZ* fusion, was chosen for further characterization, the structure of the *nucA* region of two exconjugants for each construct was confirmed by Southern analysis (data not shown). Like the parental strain PCC 7120, these strains were able to develop heterocysts and to grow on N_2 (data not shown).

Analysis of the *lacZ*-carrying strains showed that only a low activity of β-galactosidase, 0.5 ± 0.02 mU · mg of protein^{-1}, was produced in the absence of a promoter fragment (strain bearing the control construct from plasmid pCSAV50). Incorporation of a *glnA* upstream fragment, fragment C, B, or A (Fig. 3), resulted in β-galactosidase activities of 5.1 ± 1.1 mU · mg of protein^{-1} (fragment C), 1.7 ± 0.46 mU · mg of

protein^{-1} (fragment B), and 3.8 ± 0.7 mU · mg of protein^{-1} (fragment A). These results indicated that there was promoter activity of the DNA fragments tested, but the β-galactosidase activities exhibited by the different strains should not be compared to each other, since the promoter fragments generated transcripts with different 5′ regions that may have had different stabilities and/or translation efficiencies. On the other hand, β-galactosidase activities driven by fragments A, BA, and CBA or by fragments B and CB can be compared. Fragment BA drove a β-galactosidase activity of 12.2 ± 1.9 mU · mg of protein^{-1}, which is higher than the activity driven by fragment A. In contrast, fragments CB (1.5 ± 0.56 mU · mg of protein^{-1}) and CBA (10.6 ± 2.3 mU · mg of protein^{-1}) directed activities that were similar to those produced by fragments B and BA, respectively.

Mutation of the NtcA-binding site. As shown above, use of the P_1 promoter that generates RNA_I is dependent on NtcA (15). This promoter carries an NtcA-binding site ($GTAN_8TAC$) that is centered at position −41.5 with respect to the transcription start point. To characterize the role of this NtcA-binding site in transcription from the *glnA* promoter, we generated a mutated CBA fragment (CBA*) in which the GTA triplet of the NtcA-binding site was replaced by a CAT triplet (see Materials and Methods for details). This mutated fragment was incorporated into the *lacZ* reporter system and transferred to *Anabaena* sp. strain PCC 7120, where it directed a β-galactosidase activity of 154.8 ± 6.7 mU · mg of protein^{-1}, which is about 15-fold higher than the activity observed with the original CBA fragment.

To test the effect of mutation of the NtcA-binding site on the use of the *glnA* promoter(s), primer extension assays were performed with a primer from the *lacZ* upstream region and RNA isolated from ammonium-grown filaments incubated for 9 h in the absence of combined nitrogen. After transfer of the CBA::*lacZ* and CBA*::*lacZ* constructs to strain CSE2 (*ntcA*), these experiments were carried out in the wild-type and *ntcA* genetic backgrounds. The CBA fragment produced a set of primer extension products that included the same RNA species that were observed with the native *glnA* promoter (Fig. 4A, lane 1) (RNA_V was not shown in this experiment). However, RNA_I was not observed in an *ntcA* mutant background (Fig. 4A, lane 2). These results confirmed the proper operation as a promoter of fragment CBA cloned in front of *lacZ*. When the mutated CBA fragment (fragment CBA*) was analyzed, transcription was observed to originate mainly from a transcription start point corresponding to RNA_{II}, and RNA_I was detected neither in the wild type (Fig. 4B, lane 2) nor in the *ntcA* background (Fig. 4B, lane 3).

Binding of NtcA. The *glnA* P_1 promoter bears a very efficient NtcA-binding site (37). To test other possible NtcA interactions with the *Anabaena glnA* upstream region used in this work, binding of NtcA to the different promoter fragments was tested by a band shift assay. Binding was observed as long as the sequences corresponding to P_1 were present in the DNA fragment tested but did not take place when the NtcA-binding site in P_1 was mutated (Fig. 5). DNase I footprinting of a DNA fragment covering the whole *glnA* upstream region that we investigated (fragment CBA) did not show any NtcA-sensitive region in addition to the region previously described by Ramasubramanian et al. (37), which corresponds to the NtcA-binding site in the P_1 promoter (results not shown).

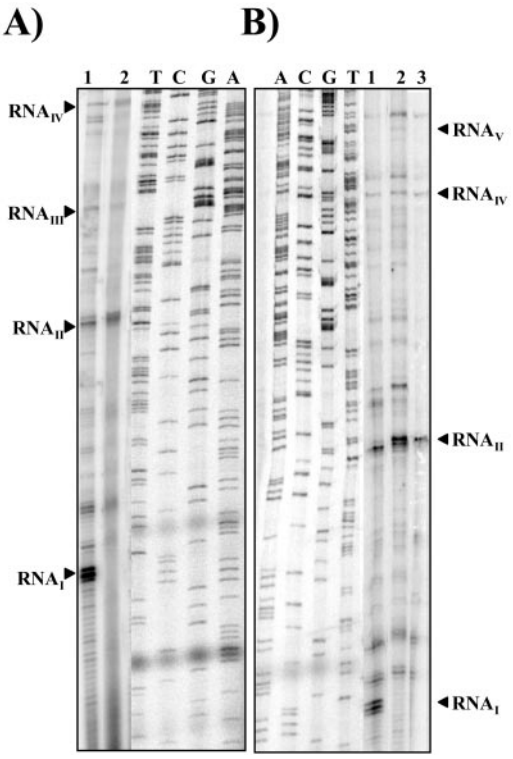

FIG. 4. Transcription start points for the CBA and CBA* P_{glnA}::*lacZ* fusions in *Anabaena* sp. strains PCC 7120 (wild type) and CSE2 (*ntcA*). Primer extension analysis was carried out with primer LZ3 (Fig. 3B), and a sequencing ladder was generated with plasmid pCSAV127 and the same primer. (A) RNA isolated from filaments of strains PCC 7120 (lane 1) and CSE2 (lane 2) carrying the CBA::*lacZ* fusion grown with ammonium and incubated for 9 h with no source of combined nitrogen. (B) RNA isolated from filaments of strain PCC 7120 carrying CBA::*lacZ* (lane 1) or CBA*::*lacZ* (lane 2) or of strain CSE2 carrying CBA*::*lacZ* (lane 3) grown with ammonium and incubated for 9 h with no source of combined nitrogen.

DISCUSSION

The *glnA* gene of *Anabaena* sp. strain PCC 7120 appears to be transcribed from four independent transcription start points, which give rise to the RNA species known as RNA_I, RNA_{II}, RNA_{IV}, and RNA_V (Fig. 1). These four RNA species are observed in ammonium-grown *Anabaena* filaments; however, whereas RNA_{II} appears to be constitutive, RNA_I, RNA_{IV}, and RNA_V are induced after nitrogen deprivation (15; this study). DNA fragments carrying sequences upstream from RNA_I (fragment A), RNA_{II} (fragment B), and RNA_{IV} (fragment C) drive transcriptional activity when they are fused to a *lacZ* reporter gene, demonstrating that these RNA species define true gene promoters (P_1, P_2, and P_3, respectively). Fragment C also covers RNA_V, and therefore an additional promoter, P_4, may be present in this DNA fragment. Fragment BA produces higher β-galactosidase activity than fragment A, which, consistent with detection of RNA_{II} and RNA_I in samples of RNA isolated from whole filaments, suggests that there

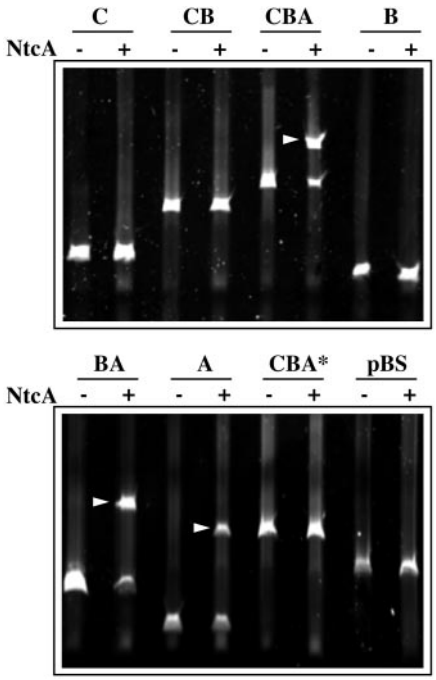

FIG. 5. Band shift assays of the binding of NtcA to the *Anabaena glnA* upstream region. The DNA fragments indicated at the top were obtained by PCR, and 75 to 100 ng of each of them was used in binding assays with extracts of *E. coli* carrying the NtcA-overproducing plasmid pCSAM70 (lanes +) or, as a control, the pQE9 vector (lanes −). The arrowheads indicate retarded fragments, which were observed only with the A, BA, and CBA fragments and the NtcA-containing extract. pBS, DNA fragment from pBluescript SK(+) used as a negative control.

is simultaneous operation of the P_2 and P_1 promoters. In contrast, although fragment C itself drives a substantial β-galactosidase activity and RNA_{IV} and RNA_V have been observed in different RNA samples, fragments CB and CBA do not direct higher levels of activity than fragments B and BA, respectively. The reason for this lack of stimulation of β-galactosidase production by fragment C is unknown, but the presence in fragment B of the putative RNA processing site that generates RNA_{III} (Fig. 1 and 3), which could interfere with transcription driven by promoters P_3 and P_4, should be noted.

In heterocysts, expression takes place essentially as RNA_I, indicating that P_1 represents the *glnA* promoter in this cell type (Fig. 2A). A transcript corresponding to RNA_I has also been shown to represent the main *glnA* transcript during heterocyst differentiation (49) or in cells starved for nitrogen under anaerobic conditions (45). However, P_1 is not a promoter that is used exclusively in the heterocysts since it is also utilized in vegetative cells (i.e., in cells of filaments grown with ammonium) (Fig. 2A) and in a *hetR* mutant (a strain that does not develop heterocysts) (Fig. 2B). P_1 represents a canonical NtcA-type promoter whose use is strictly dependent on NtcA (Fig. 2B and 4A) (15). Mutation of the NtcA-binding site in P_1 through replacement of the GTA triplet by CAT eliminates NtcA binding in vitro (Fig. 5) and the use of P_1 in vivo (Fig. 4B), indicating the importance of this NtcA-binding site for NtcA-dependent transcription activation. The amount of NtcA present in vegetative cells (34, 38) appears to determine levels of binding of NtcA to the efficient NtcA-binding site in P_1 that are sufficient for the use of P_1 in this type of cells, while the high levels of active NtcA likely present in the heterocysts (34, 37, 49) should contribute to strong use of P_1 in these differentiated cells.

P_2 represents a canonical σ^{70}-type promoter (Fig. 1) that is used in vegetative cells but not in heterocysts (Fig. 2A). The presence of the NtcA-binding site in P_1 appears to affect the operation of P_2. Thus, mutation of the NtcA-binding site in P_1 results in a dramatic increase in β-galactosidase activity and in increased transcription from P_2 (Fig. 4B). However, when results with the CBA and CBA* fragments are compared, the increase in RNA_{II} levels detected by primer extension analysis is not quantitatively comparable to the observed increase in β-galactosidase activity, suggesting that, in addition to transcriptional activity, transcript stability or translation efficiency may affect the production of β-galactosidase. Nonetheless, because the NtcA-binding site in P_1 (footprinted region in Fig. 1) is 9 bp downstream of the RNA_{II} transcription start point, NtcA bound to this site could repress P_2 either by occluding RNA polymerase binding or by inhibiting promoter escape (29, 40). Repression by NtcA might therefore contribute to the lack of use of P_2 in heterocysts. However, increased transcription from P_2 is hardly observed when the *ntcA* gene, rather than the NtcA-binding site, is inactivated (Fig. 2B and 4A). Apart from the possible repressor role of NtcA, it is possible that the NtcA-binding sequence itself interferes with the use of P_2.

Promoters P_3 and P_4 are positively influenced by NtcA, although sequence scrutiny does not permit identifying any of these promoters as an NtcA-type promoter. NtcA-dependent promoters that cannot be recognized as NtcA-type promoters have also been described for some other *Anabaena* genes, suggesting that there is indirect operation of NtcA. This is the case for the *hetR* gene, which in *Anabaena* sp. strain PCC 7120 is transcribed from four promoters, two of which are NtcA dependent but do not contain sequences matching the consensus sequence for NtcA-activated promoters (34). Lack of binding of NtcA to fragment C (Fig. 5) is consistent with indirect regulation by NtcA of the *glnA* P_3 and P_4 promoters.

To summarize, expression of the *glnA* gene, which encodes the key nitrogen assimilation enzyme glutamine synthetase, takes place in *Anabaena* sp. strain PCC 7120 from a set of promoters whose utilization is influenced by the global N-control transcription factor NtcA. Whereas the utilization of P_1 is strictly dependent on the binding of NtcA to this promoter, operation of promoters P_3 and P_4 appears to be indirectly dependent on NtcA. In contrast, the NtcA-binding site in P_1 interferes with transcription from the P_2 promoter.

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