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 \textit{glnA} gene encoding glutamine synthetase is subject to a variety of regulatory mechanisms in different organisms. In the filamentous, \textit{N}_2-fixing cyanobacterium \textit{Anabaena} sp. strain PCC 7120, \textit{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\textsubscript{I} originates from a canonical NtcA-dependent promoter (P\textsubscript{1}), RNA\textsubscript{II} originates from a \(\sigma^70\)-type promoter (P\textsubscript{2}). RNA\textsubscript{IV}, which is influenced by NtcA but the corresponding promoter (P\textsubscript{3}) does not have the structure of NtcA-activated promoters. Using RNA isolated from \textit{Anabaena} filaments grown under different nitrogen regimes, we observed, in addition to these transcripts, RNA\textsubscript{V}, which has previously been detected only in vitro transcription assays and should originate from P\textsubscript{4}. However, in heterocysts, which are differentiated cells specialized in \textit{N}_2 fixation, RNA\textsubscript{V} was the almost exclusive \textit{glnA} transcript. Analysis of \textit{P}_\textit{ntcA}\textit{lacZ} fusions containing different fragments of the \textit{glnA} upstream region confirmed that fragments carrying \textit{P}_1, \textit{P}_2, or \textit{P}_3 and \textit{P}_4 have the ability to promote transcription. Mutation of the NtcA-binding site in \textit{P}_1 eliminated \textit{P}_1-directed transcription and allowed increased use of \textit{P}_4. The NtcA-binding site in the \textit{P}_4 promoter and binding of NtcA to this site appear to be key factors in determining \textit{glnA} gene expression in vegetative cells and heterocysts.

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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 than in the absence of ammonium than in the presence of ammonium (45), is NtcA dependent, and originates from a promoter (that we call P1) with the canonical structure of the NtcA-dependent promoters (15, 19). Indeed, NtcA binds with high efficiency to its binding site in the P1 promoter, and this NtcA-binding site has been footprinted (37). RNAII, whose 5′ end is located 155 (45) or 157 (15) nucleotides upstream of the start codon, is detected independently 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 RNAII, P2, 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). RNAIII, whose 5′ end has been located either 244 to 247 (45) or 196 (15) nucleotides upstream of the start translation, may arise from processing of RNAIV rather than represent a true transcription start site (42). There is some uncertainty about the exact 5′ end of RNAIV; 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 (TGTNTTAA) that could represent an extended −10 box is evident. The promoter that generates RNAIV, P3, has been described as NtcA dependent (15), although it has no evident NtcA-binding site, and it can be used in vitro transcription assays with both *Anabaena* and *E. coli* RNA polymerases (43). Finally, RNAV, 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 (P1) in ammonium-grown *Anabaena* cells and from a σ^70^-like promoter (P2, which was later identified as an NtcA-type promoter [15]) under N2-fixing conditions (45). In this work, we show that P1 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_ and 4 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH buffer (pH 7.5). For plates, the media were solidified with 15 g of agar (Difco). Cultures were grown at 30 °C in the light (75–80 μmol photons m^{-2} s^{-1}) for RNA isolation, cells growing exponentially in BG11 medium (BG11C medium plus 5 to 8 mM NH_4Cl) was grown in Luria-Bertani medium containing, when necessary, 50 μg of ampicillin·ml^{-1}, 50 μg of kanamycin·ml^{-1}, 30 μg of chloramphenicol·ml^{-1}, 25 μg of streptomycin·ml^{-1}, or 100 μg of tetracycline·ml^{-1}.

**β-Galactosidase activity.** β-Galactosidase activity was determined as previously described (46) by using chloroform-treated filaments from shaken cultures.
TABLE 1. Deoxycytidinonucleotide primers used in this work

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
</tr>
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<tbody>
<tr>
<td>GA1</td>
<td>AAGCCTTTAATGCTGATCATGCGATCC</td>
</tr>
<tr>
<td>GA2</td>
<td>CAGATATACTCACCAGACAAAGATAAGCATAC</td>
</tr>
<tr>
<td>GA3</td>
<td>GGATTTTATGCAATTTGGTAC</td>
</tr>
<tr>
<td>GA4</td>
<td>TACAGGAAGCTTCTGATTACAG</td>
</tr>
<tr>
<td>GA5</td>
<td>CGAAGAAAGGTTATATATCTG</td>
</tr>
<tr>
<td>GA6</td>
<td>CGAAGAAAGTTGTGAGTAC</td>
</tr>
<tr>
<td>GA7</td>
<td>CTTTCAGAAGCTTCTTGTGGG</td>
</tr>
<tr>
<td>GA8</td>
<td>CAAGAGATCTCATTACGAAGCTAC</td>
</tr>
<tr>
<td>GA10</td>
<td>GTATCTTTGTATGAGAAACGTCTG</td>
</tr>
<tr>
<td>GA13</td>
<td>CTCTCTCTCGCAATTTTC</td>
</tr>
<tr>
<td>LZ3</td>
<td>CAATCAGCTGCTCAATGCCC</td>
</tr>
</tbody>
</table>

that were grown in BG11 medium containing NH₄Cl and incubated for 3 days in BG11 medium with air levels of CO₂. 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 P₁ promoter. The deoxycytidinonucleotide primers used in this work are shown in Table 1. Site-directed mutagenesis of the P₁ 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, pCSAV17, 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, A, and CBA*, respectively (see Fig. 3). The amplified products were cloned in vector pGEM-T (Promega). SphII/Sall fragments from the resulting plasmids (containing the cloned fragment) and from plasmid pCSAV17 (which bears fragment CBA*) were inserted between SphII and Sall sites in vector pC20R (25). A 2.4-kb Sm restriction fragment with HindIII ends was inserted into the unique HindIII site of pC20R. A BamHI fragment from pPE20 containing the promoterless lacZ gene (26) was cloned into a BamHI site just after the glnA promoter fragment. A plasmid without a 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 Klnow enzyme (this inactivation was necessary because the increase in nucA expression directed from the Km’ cassette inserted upstream of this gene could be lethal for the E. coli host). BglII fragments containing the Sm’ Sp’ 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, 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 transfected by conjugation (52) to Anabaena sp. strain PCC 7120 and, as indicated below, also to strain CNE2 (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 T7 Sequencing kit (Amersham Biosciences) and [α-32P]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, hybridization in 5% formamide and 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 using 0.1 M NaOH hybridization was performed at 65°C according to the recommendations of the manufacturers of the membranes. The nfiI and rblc probes were internal fragments of these genes amplified by PCR. All probes were 32P labeled with a Ready to Go DNA labeling kit (Amersham Biosciences) by using [α-32P]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 pCSAV44, oligonucleotide LZ3 (complementary to a sequence located upstream of the lacZ gene) was used as the primer, and plasmid pCSAV172 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 pCSAV17 were used for PCR amplification of the glnA upstream region (wild-type and mutated versions, respectively). Oligonucleotides (pUCM13 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(pOED9, pREP4).

The DNase I protection assay was carried out as described previously (16). The DNA was used as the insert of pCSAV26 (containing fragment CBA in the pC20R vector) limited by BamHI/EcoRRII or HindIII-Sacl sites and 3′ end labeled with the DNA polymerase Klenow fragment and [α-32P]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 N₂-fixing filaments. This RNA preparation did not show any detectable hybridization with an rblc probe, whereas it exhibited strong hybridization with a nfiI 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 N₂ 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 274 (RNA274) 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 nucleo-
otides upstream of the start codon (corresponding to RNA_{III}) 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 start codon (corresponding to 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_{III} 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) and, as controls, from strain CSE2 (ntcA) and, wild-type strain PCC 7120 were used. Whereas in the hetR mutant RNA_{III} was not detectable and RNA_{IV} was in-

duced 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_{IV} 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 0.3 ± 0.1 mU · mg of protein^{-1} (fragment A).
protein \(^{-1}\) (fragment B), and \(3.8 \pm 0.7 \text{ mU} \cdot \text{mg of protein}^{-1}\) (fragment A). These results indicated that there was promoter activity of the DNA fragments tested, but the \(\beta\)-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, \(\beta\)-galactosidase activities driven by fragments A, BA, and CBA by or fragments B and CB can be compared. Fragment BA drove a \(\beta\)-galactosidase activity of \(12.2 \pm 1.9 \text{ mU} \cdot \text{mg of protein}^{-1}\), which is higher than the activity driven by fragment A. In contrast, fragments CB (\(1.5 \pm 0.56 \text{ mU} \cdot \text{mg of protein}^{-1}\)) and CBA (\(10.6 \pm 2.3 \text{ mU} \cdot \text{mg of protein}^{-1}\)) 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 RNAI is dependent on NtcA (15). This promoter carries an NtcA-binding site (GTANATAC) 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 \(\beta\)-galactosidase activity of \(154.8 \pm 6.7 \text{ mU} \cdot \text{mg of protein}^{-1}\), 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) (RNAIV was not shown in this experiment). However, RNAI 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 RNAII, and RNAI 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).

**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 RNAI, RNAII, RNAIV, and RNAV (Fig. 1). These four RNA species are observed in ammonium-grown *Anabaena* filaments; however, whereas RNAII appears to be constitutive, RNAI, RNAIV, and RNAV are induced after nitrogen deprivation (15; this study). DNA fragments carrying sequences upstream from RNAI (fragment A), RNAII (fragment B), and RNAIV (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 RNAV, and therefore an additional promoter, \(P_4\), may be present in this DNA fragment. Fragment BA produces higher \(\beta\)-galactosidase activity than fragment A, which, consistent with detection of RNAI, and RNAII in samples of RNA isolated from whole filaments, suggests that there
of binding of NtcA to the efficient NtcA-binding site in P1 that are sufficient for the use of P1 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 P1 in these differentiated cells.

P2 represents a canonical σ70-type promoter (Fig. 1) that is used in vegetative cells but not in heterocysts (Fig. 2A). The presence of the NtcA-binding site in P1 appears to affect the operation of P2. Thus, mutation of the NtcA-binding site in P1 results in a dramatic increase in β-galactosidase activity and in increased transcription from P2 (Fig. 4B). However, when results with the CBA and CBA* fragments are compared, the increase in RNAIII 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 P1 (footprinted region in Fig. 1) is 9 bp downstream of the RNAIII transcription start point, NtcA bound to this site could repress P2 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 P2 in heterocysts. However, increased transcription from P2 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 P2.

Promoters P3 and P4 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 P3 and P4 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 P1 is strictly dependent on the binding of NtcA to this promoter, operation of promoters P3 and P4 appears to be indirectly dependent on NtcA. In contrast, the NtcA-binding site in P3 interferes with transcription from the P2 promoter.

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**REFERENCES**

