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Transcription of the *Anabaena* sp. Strain PCC 7120 *ntcA* Gene: Multiple Transcripts and NtcA Binding

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The *Anabaena* sp. strain PCC 7120 *ntcA* gene showed multiple transcripts with different 5′ ends. The relative abundance of transcripts varied in response to nitrogen availability. The *ntcA* product, NtcA, showed binding to the promoter region of its own gene. The binding site mapped to a region between the transcription start site used under nitrogen-replete conditions and the start sites used under nitrogen-limiting conditions, suggesting that NtcA regulates its own expression.

NtcA is a DNA-binding protein whose predicted amino acid sequence shows similarity to those of the family of prokaryotic regulatory proteins represented by the cyclic AMP receptor protein, CRP (19, 22). An *ntcA* mutant of *Anabaena* sp. strain PCC 7120 requires ammonium for growth and is defective for heterocyst formation (9, 21), indicating that NtcA is required for utilization of nitrate and dinitrogen. Besides being involved in nitrate assimilation and heterocyst differentiation, NtcA interacts with *xisA*, *glnA*, *rbcL*, and *nifH* promoter fragments in vitro (17). NtcA (BifA or VF1) was identified in PCC 7120 on the basis of its interaction with the *xisA* upstream region (6). NtcA binds to three sites upstream of *xisA*, which encodes a site-specific recombinase involved in the excision of an 11-kb DNA element from the chromosome during heterocyst development (6, 11, 13). The NtcA binding site is about 30 bp from a recombination site, suggesting that NtcA is involved in the excision of the 11-kb element. In the *glnA* promoter, NtcA binds strongly to a single site located between the major transcription start site used in vegetative cells (RNA_{II}) and the major transcription start site used under nitrogen-limiting conditions (RNA_I) (17, 18). Furthermore, Northern (RNA) and primer extension analyses of RNA from an *ntcA* mutant show that NtcA is required for transcription from the *glnA* RNA_I promoter (9, 21).

The predicted amino acid sequence of the PCC 7120 *ntcA* gene is 77% identical to that of the *Synechococcus* sp. strain PCC 7942 *ntcA* gene (19, 22). NtcA is required for the full expression of genes subject to ammonium repression in PCC 7942 (20). Through protein-DNA interactions, NtcA positively regulates the expression of the *nir* and *glnA* genes during growth on nitrate as well as after nitrogen starvation (7, 15). NtcA has also been shown to bind upstream of its own gene and autoregulate its expression in PCC 7942 (15).

Here we show that multiple transcripts of *ntcA* are produced under nitrogen-limiting conditions in PCC 7120. We also show that NtcA protein binds upstream of the *ntcA* gene, suggesting that NtcA regulates its own expression.

Transcription of *ntcA*. Since *ntcA* is required for nitrate assimilation and heterocyst formation (9, 21), we analyzed the expression of *ntcA* in media containing different nitrogen sources. *Anabaena* sp. strain PCC 7120 was grown in 100 ml of BG-11 medium (12) containing either no combined nitrogen source, 5 mM NH_4NO_3, 10 mM $NaNO_3$, or 5 mM NH_4Cl for 3 days (A_{750}, 0.11 to 0.15) or 6 days (A_{750}, 0.50 to 0.56). RNA was prepared from filaments by an acidic hot phenol method (16, 21), which we have shown to extract RNA from vegetative cells and heterocysts (unpublished data). RNA samples (10 μg) were transferred from formaldehyde-agarose gels to MagnaCharge membranes, and Northern blots were hybridized with a random-primer-labeled probe as previously described (12, 21). An 824-bp *Eco*RI fragment from pAM997 (21) containing *ntcA* was used as the probe.

ntcA produced multiple transcripts under nitrogen-limiting conditions (Fig. 1). A 0.8-kb transcript was seen both in the presence and absence of combined nitrogen and regardless of the source of combined nitrogen, suggesting that *ntcA* is constitutively transcribed. These results are consistent with two recent findings. NtcA DNA-binding activity is present in cells grown under nitrogen-replete conditions as well as nitrogen-limiting conditions (17), and transcriptional fusion of the *ntcA* upstream region to dual reporters (promoterless CAT-promoterless *nifHDK*) in an *nifH* mutant of PCC 7120 showed that *ntcA* is expressed under both nitrogen-replete (resistance to chloramphenicol) and nitrogen-limiting (*nifHDK* expression) conditions (2). RNAs from filaments grown in the presence of combined nitrogen, especially from the 6-day samples, showed the presence of longer transcripts (up to 1.4 kb in length), as has been previously observed (21).

In addition to the 0.8-kb *ntcA* transcript, cultures grown without combined nitrogen produced a 1-kb *ntcA* transcript (Fig. 1). This transcript was seen with both 3-day (log-phase) and 6-day (late-log–early-stationary-phase) cultures. This result may be related to the findings that *ntcA* transcription increases upon nitrogen step-down (21) and that NtcA DNA-binding activity is higher in isolated heterocysts (17).

***ntcA* has multiple putative transcription start sites.** Analysis of *ntcA* RNA by primer extension showed multiple 5′ ends, suggesting multiple transcription start sites in the absence of combined nitrogen (Fig. 2 and 3). Total RNA was prepared from PCC 7120 cells grown for 6 days in 100 ml of BG-11 medium without or with different sources of combined nitrogen. RNA was also isolated from filaments collected at 6-h intervals during large-scale heterocyst induction of PCC 7120 (11, 21). In this heterocyst induction experiment, proheterocysts were present at 12 h and mature heterocysts containing polar granules were present at 15 to 16 h after nitrogen step-

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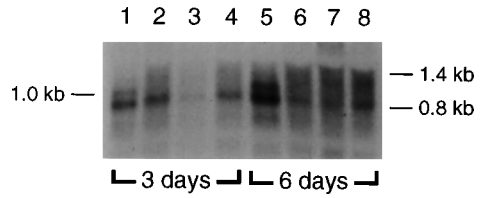

FIG. 1. Northern analysis of the *ntcA* gene. Total RNA was isolated from wild-type PCC 7120 cells grown for 3 (lanes 1 through 4) or 6 (lanes 5 through 8) days without a combined nitrogen source (lanes 1 and 5) or grown with 5 mM NH_4NO_3 (lanes 2 and 6), 10 mM $NaNO_3$ (lanes 3 and 7), or 5 mM NH_4Cl (lanes 4 and 8). The RNA blot (10 μg RNA per lane) was hybridized with a labeled *ntcA* probe. The sizes of major transcripts are indicated.

down. A similar induction was done to prepare isolated heterocysts, which were purified by passing filaments through a Stansted cell disrupter (5), and RNA was extracted and purified by centrifugation through 5.7 M CsCl (21). Approximately 20 μg of RNA from the small-scale induction, 25 μg of RNA from time course samples, and 17 μg of RNA from heterocysts were used for cDNA synthesis. Primers (100 ng) A, B, and C (Fig. 2) were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled primers were annealed to RNA and extended with avian myeloblastosis virus reverse transcriptase at 42°C for 1 h, and the cDNA was analyzed on a 6% polyacrylamide–urea gel (1).

Primer extension with primer A indicated a major *ntcA* transcript with a 5′ end at −49 (Fig. 3A). This transcript was present in all samples, regardless of the source of combined nitrogen; however, the transcript was more abundant in samples from medium lacking a source of combined nitrogen. RNA from a heterocyst induction time course showed an interesting pattern. After a peak of *ntcA* expression at 12 h, there was a drop at 18 h, followed by a gradual increase in expression later. This pattern parallels the *ntcA* expression seen in Northern blots during this time course (21). In heterocysts, this transcript was abundant. The strong expression of *ntcA* during the early stages of heterocyst differentiation and the increased NtcA DNA-binding activity in heterocysts (17) suggest that NtcA plays an important role in heterocyst differentiation and are consistent with the observation that the *ntcA* mutant is Het$^-$ (21). In addition to the 5′ end at −49, a 5′ end at −180 was detected in RNA isolated at the 12-h time point.

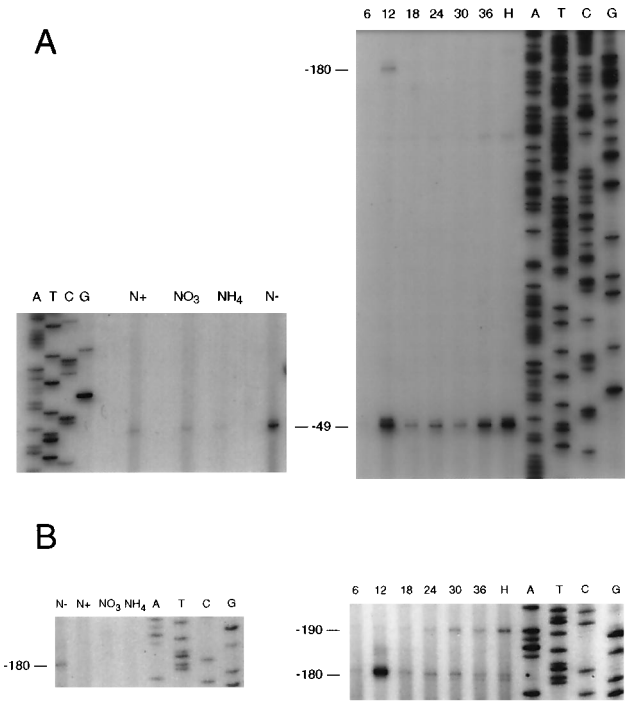

FIG. 3. Identification of the 5′ ends of *ntcA* transcripts. Total RNA from 100-ml cultures of wild-type PCC 7120 cells grown for 6 days in the absence of a combined nitrogen source (N−) or grown with 5 mM NH_4NO_3 (N+), 10 mM $NaNO_3$ (NO_3), or 5 mM NH_4Cl (NH_4); from filaments collected at 6, 12, 18, 24, 30, and 36 h after nitrogen step-down; or from heterocysts free of vegetative cells (H) was hybridized to ^{32}P-end-labeled primer A (A) or B (B), subsequently extended with reverse transcriptase, and analyzed on a 6% polyacrylamide–urea gel. The fragments are labeled to indicate putative transcription start sites with respect to the ATG translation initiation codon, as shown in Fig. 2. Lanes A, T, C, and G are dideoxy sequencing ladders of pAM997 produced by primers A and B.

To confirm the 5′ end of the *ntcA* transcript at −180, we designed a second primer, B (Fig. 2), that is closer to this position. The extension of primer B showed a 5′ end that mapped to −180 in RNA from cultures lacking combined nitrogen, but not in RNA from cultures containing a source of combined nitrogen (Fig. 3B). RNA from a heterocyst induction time course showed two 5′ ends with primer B. The expression pattern of the *ntcA* transcript mapping to −180 paralleled that of the transcript at −49, peaking at 12 h. Besides the 5′ end seen at −180, a third 5′ end was present in RNA isolated during the induction time course. The expression pattern of the *ntcA* transcript mapping to −190 differed from those of the transcripts mapping to −49 and −180. The −190 *ntcA* transcript increased in expression at later time points during heterocyst induction and was abundant in heterocysts.

The presence of the 5′ ends at −180 and −190 was confirmed with a third primer, C (Fig. 2). The 5′ end mapping

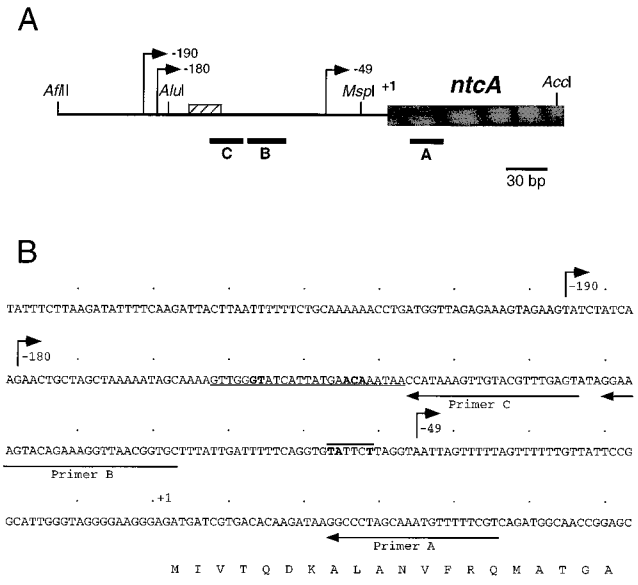

FIG. 2. Map of the *ntcA* upstream region. (A) The gray box indicates the 5′ end of the *ntcA* coding sequence, and the start of the gene is designated +1. Arrows indicate putative transcription start sites at −49, −180, and −190. Black boxes labeled A, B, and C indicate the positions of the primers used for primer extension analysis. The hatched box marks the position of the NtcA binding site. (B) Nucleotide sequence of the *ntcA* upstream region. The NtcA binding site identified by DNase I protection is underlined, and the conserved NtcA recognition sequence is in bold. The primers and the putative transcription start sites at −49, −180, and −190 are labeled. A potential −10 consensus sequence upstream of the −49 transcription start site is overlined, and its highly conserved bases are in bold.

results with primer C were identical to those shown in Fig. 3B. Other faint bands seen with primer A (Fig. 3A) could not be confirmed with primer B or C, suggesting that they were experimental artifacts.

Sequences upstream of the −49 transcription start site are similar to the −10 consensus for *Anabaena* sp. strain PCC 7120 vegetative-cell promoters (Fig. 2) and partially resemble the *Escherichia coli* σ^{70} promoter consensus −10 hexamer (8). No similarity to a consensus promoter was found upstream of the −180 and −190 start sites. The fact that −180 and −190 transcripts are present only under nitrogen-limiting conditions helps explain the observation of Wei et al. that strain AMC273, which carries the *ntcA* gene and 87 bp of the upstream region in pAM1322, grew in the presence of nitrate as the sole source of combined nitrogen but was unable to grow without combined nitrogen (22). In contrast, strain AMC274, which carries the *ntcA* gene and 251 bp of the upstream region in pAM1323, grew under both nitrogen-replete and nitrogen-limiting conditions (22). These in vivo results suggest that the expression of *ntcA* from the −180 and −190 transcription start sites is important for growth under nitrogen-limiting conditions.

***ntcA* promoter activity in vivo.** To determine if the *ntcA* upstream region containing the putative transcription start sites showed promoter activity in vivo, we constructed transcriptional fusions between this region and a promoterless *lacZ* gene (5) in the shuttle vector pAM504 (21). Strains of PCC 7120 containing various plasmids were grown to mid-log phase under nitrogen-limiting conditions, and protein extracts were assayed for β-galactosidase activity (14). In a representative experiment, pAM1398, which contains the region from −171 to −21, produced 84 U of β-galactosidase specific activity (in nanomoles of *o*-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein) and pAM1399, which contains the region from −251 to −117, produced 230 U. In contrast, pAM733, which contains the promoterless *lacZ* gene alone, produced no β-galactosidase activity; pAM1401, which contains the region from −171 to −21 cloned in the reverse orientation, produced only 5 U of activity. Qualitatively similar results were obtained when the same strains were grown in medium containing NH_4NO_3. These results show that the *ntcA* −49 and −180/−190 upstream regions have promoter activities and suggest that the RNA 5′ ends identified by primer extension represent transcription start sites.

NtcA binds upstream of the *ntcA* gene. NtcA appears to be an important accessory factor that binds upstream of a number of genes in cyanobacteria (15, 17). In strain PCC 7120, NtcA has been shown to bind upstream of *glnA* and is required for the expression of *glnA* and *hetR* under nitrogen-limiting conditions (9, 17, 21). In *Synechococcus* sp. strain PCC 7942, NtcA binds upstream of *nir* and *glnA* and is required for their expression under nitrogen-limiting conditions (15, 19). PCC 7942 NtcA has also been shown to bind upstream of the *ntcA* gene in mobility shift assays (15), and examination of the DNA sequence upstream of the PCC 7120 *ntcA* gene showed two putative NtcA binding sites. Since *ntcA* shows a complex expression pattern during the growth of PCC 7120, we wanted to test whether the PCC 7120 NtcA protein binds upstream of its own gene. Partially purified NtcA was used in mobility shift assays to test its binding to a labeled *ntcA* fragment. A 385-bp *Afl*II-*Acc*I fragment (nucleotides −255 to +130 [Fig. 2]) was labeled with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. Mobility shift assays with the labeled *ntcA* fragment indicated the presence of DNA-binding activity in heparin-Sepharose fractions 9 through 15 (Fig. 4A). The elution profile of this DNA-binding activity parallels the elution profile of NtcA (17).

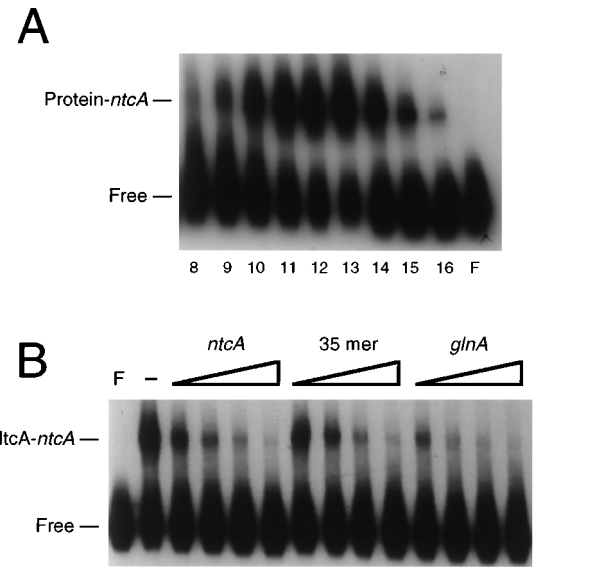

FIG. 4. NtcA binds upstream of the *ntcA* gene. (A) Mobility shift assays of a PCC 7120 vegetative-cell lysate fractionated by heparin-Sepharose chromatography. Samples (3 μl) from column fractions collected during elution with a linear (0.1 to 1.0 M) ammonium sulfate gradient were incubated with 10,000 cpm (0.8 ng) of labeled 385-bp *Afl*II-*Acc*I *ntcA* fragment and analyzed on a 5% polyacrylamide gel. The positions of the free DNA probe and protein-*ntcA* complex are shown. Lane numbers refer to the heparin-Sepharose column fractions. Lane F, free probe. (B) Competition experiment for NtcA binding to labeled *ntcA* probe. Mobility shift assays were carried out as described for panel A with or without unlabeled competitor DNA. Heparin-Sepharose column fraction 13 from panel A was used as the source of NtcA for these assays. The competitor fragments were the unlabeled *ntcA* fragment, a 35-bp oligonucleotide containing the NtcA binding site on *glnA* (35-mer), and a 249-bp *glnA* promoter fragment from pAM658. No competitor (−) or a molar excess (10-, 25-, 50-, and 100-fold) of unlabeled competitor fragment was added to samples. Lane F, free probe. The protein-DNA complex was run on a 5% polyacrylamide gel at 30 mA for 2.5 h. The positions of the free DNA probe and NtcA-*ntcA* complex are shown.

Competition experiments were done to further analyze the protein-*ntcA* complex. Mobility shift assays were done with partially purified protein extracts, the labeled *ntcA* fragment, and the following unlabeled competitors: the *ntcA* fragment, an *Xba*I-*Hin*dIII fragment from pAM658 containing 249 bp of the *glnA* promoter region, and a 35-bp oligonucleotide containing the *ntcA* binding site on *glnA* (Fig. 4B). The autoradiographs were scanned, and the amount of binding complex formed was quantitated with MacBAS 2.0 (Fujix) software. A 25-fold excess of the unlabeled *ntcA* fragment reduced the binding to 21%, and a 100-fold excess reduced the binding to 6%. Similar excesses (25- and 100-fold) of the 35-bp fragment caused similar reductions in binding. A 25-fold excess of the unlabeled *glnA* fragment reduced the binding to 10% and appeared to be a better competitor than the *ntcA* fragment was. Two nonspecific competitor fragments used as controls, an *Xba*I fragment from pAM1311 containing 506 bp of the *xisC* gene (4) and a *Pvu*I-*Sca*I fragment from pBluescript II SK(+) containing 110 bp of vector sequence, did not significantly reduce the complex formed with the labeled *ntcA* fragment (data not shown). These experiments showed that both the *glnA* fragment and the oligonucleotide compete with the *ntcA* upstream region for binding, strongly suggesting that the protein which binds the *ntcA* upstream region is NtcA.

To confirm that NtcA and not a minor contaminant is responsible for this DNA-binding activity, we tested a protein extract from the *ntcA* mutant strain AMC236 (21) for its ability

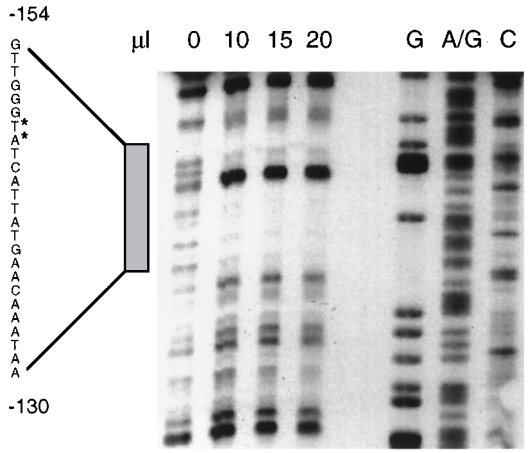

FIG. 5. DNase I footprint of the NtcA binding sites on *ntcA*. Approximately 10,000 cpm of a labeled *Eco*RI-*Sal*I fragment of pAM1382 containing the *ntcA* upstream region (from −21 to −171 bp with respect to the ATG codon) was incubated with the indicated amounts of NtcA-containing heparin-Sepharose column fractions (0.1 μg of total protein μl^{-1}), digested with 1 μl of 40-U/ml DNase I for 2 min, and analyzed on a 6% polyacrylamide–urea gel. Lanes G, A/G, and C are Maxam-Gilbert sequencing ladders produced from the labeled fragment. The gray box marks the DNase I-protected region. Nucleotide numbers are with respect to the translation start site. Asterisks indicate DNase I-hypersensitive sites.

to bind the labeled *ntcA* fragment in a mobility shift assay. Unlike wild-type protein extracts, the AMC236 extract failed to form a complex with the *ntcA* upstream region (data not shown). Furthermore, NtcA produced from the PCC 7120 *ntcA* gene cloned in *E. coli* formed a complex with the labeled *ntcA* fragment. Mobility shift assays with protein extracts from *E. coli* harboring pAM996, which contains the *ntcA* gene (22), showed a DNA-protein complex with the same mobility as that produced by wild-type PCC 7120 extracts (data not shown). Control extracts from *E. coli* harboring only the vector plasmid failed to form a similar complex. These experiments show that the *ntcA* gene product binds upstream of its own gene.

NtcA binding site. To localize the NtcA binding site on *ntcA*, an *Eco*RI-*Sal*I fragment from pAM1382 that contains the *Alu*I-*Msp*I fragment of *ntcA* (Fig. 2) cloned into the *Cla*I-*Eco*RV site of pBluescript II KS(+) was used in mobility shift assays with partially purified NtcA. The labeled pAM1382 fragment, which contains the *ntcA* upstream sequence from −171 to −21, formed a protein-DNA complex similar to that of the larger 385-bp *ntcA* fragment (Fig. 4A).

The *ntcA* fragment from pAM1382 was used in DNase I footprinting experiments to identify the NtcA binding site (Fig. 5). The *Eco*RI-*Sal*I fragment, labeled on the coding strand, was incubated with partially purified NtcA, digested with DNase I, and analyzed on a 6% polyacrylamide–urea gel as described previously (17). The region from −130 to −154 bp upstream of the translation start site was protected from DNase I (Fig. 5). This protected region is between the two putative *ntcA* transcription start sites and extends from 25 nucleotides downstream of the −180 transcription start site to 83 nucleotides upstream of the −49 transcription start site (Fig. 2). The labeled coding strand showed DNase I-hypersensitive sites at −147 and −148, which indicates perturbation of the DNA backbone. The NtcA binding sites on *rbcL* and *glnA* also contain DNase I-hypersensitive sites (17). This suggests that NtcA, like CRP, an archetypal DNA-bending protein (10), probably causes the bending of DNA. The derived consensus sequence from NtcA binding sites on *glnA*, *xisA*, and *rbcL*, TGT($N_{9–10}$)ACA (17), was mostly conserved in the NtcA binding sequence, GT(N_{10})ACA, upstream of the *ntcA* gene.

Conclusion. NtcA appears to be an important accessory transcription factor that is involved in a number of processes in PCC 7120, such as nitrate assimilation and heterocyst differentiation (9, 21). It has been shown to bind upstream of a number of genes, including *rbcL*, *glnA*, *xisA*, and *nifH* (17), and appears to play a role in the expression of *glnA* and *hetR* (9, 21). Northern analysis and 5′ end mapping of *ntcA* RNA suggest that *ntcA*, like *glnA* and *hetR*, has multiple transcripts and that some of these transcripts are present only under nitrogen-limiting conditions (3, 18). NtcA binds upstream of *glnA* and is required for the expression of *glnA* from the RNA_I promoter under nitrogen-limiting conditions, and *hetR* expression does not increase under nitrogen-limiting conditions in an *ntcA* mutant (9, 21). The results presented here indicate that NtcA binds upstream of its own gene and that the binding site maps to a region between the *ntcA* −49 and −180/−190 putative transcription start sites. Taken together, these results show that the *Anabaena* sp. strain PCC 7120 *ntcA* gene has a complex expression pattern and may be regulated by its own gene product, as has been shown for *ntcA* in strain PCC 7942 (15).

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