NtcA Regulates *patA* Expression in *Anabaena* sp. Strain PCC 7120[∇]

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***patA* expression is induced 3 to 6 h after nitrogen step-down. We establish that the transcription of *patA* is under the positive control of NtcA. The *patA* promoter region shows two conserved NtcA-binding boxes. These NtcA-binding sites and their interaction with NtcA are key elements for *patA* expression in heterocysts.**

In response to nitrogen deficiency, the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 develops the heterocyst, a terminally differentiated cell type, specialized in the fixation of atmospheric nitrogen (9). Two major regulators control this differentiation program (NtcA and HetR) (1, 2). The accumulation of 2-oxoglutarate (2-OG) is the intracellular signal initiating the cell differentiation process; at a threshold concentration of about 0.1 mM, 2-OG stimulates the binding activity of NtcA toward its target promoters (3).

Additional factors are involved to ensure periodic heterocyst patterning (10). Among these factors, PatA has been shown to positively influence heterocyst formation. The *patA* mutant differentiates heterocysts mostly at the ends of the filaments, suggesting that the PatA protein must be required for the formation of intercalary heterocysts (5). Since the *patA* gene is transcribed most abundantly when *Anabaena* copes with combined nitrogen starvation, it has been suggested that its expression may be under the control of a regulator related to the initiation of heterocyst differentiation (4, 5). Here we confirm this assumption and demonstrate that the transcription of *patA* is under the direct control of NtcA.

Expression of *patA* and analysis of its promoter organization. Total RNAs were isolated from *Anabaena* cells induced for heterocyst formation for 0, 2, 6, 12, and 18 h under nitrogen starvation. Transcription of *patA* was analyzed by semiquantitative reverse transcription-PCR (RT-PCR) as described previously (9) or by fusion to the green fluorescent protein (GFP). Expression of the *rnpB* gene was used as a control. RNA preparations were treated with DNase, and the efficiency of this treatment was verified by PCR. The results of RT-PCR analysis indicated that the expression of *patA* was induced between 2 and 6 h after nitrogen step-down (Fig. 1A). These results are consistent with those reported previously (5). The induction of *patA* transcription in the *ntcA* mutant was abolished (Fig. 1B). The promoter region of the *patA* gene shows

two putative consensus NtcA-binding sites (GTAN8TAC) centered around the positions –249 and –110, according to the translation start codon (Fig. 1C). The data presented in Fig. 1 suggest that the transcription of *patA* could be under the direct control of NtcA.

Binding of NtcA on the promoter of *patA*. A 430-bp DNA fragment corresponding to the *patA* promoter region was am-

FIG. 1. (A, B) Expression of *patA* in *Anabaena* sp. strain PCC 7120. Semiquantitative RT-PCR analysis of *patA* and *rnpB* transcripts. Total RNAs were isolated from wild-type (A) or *ntcA* mutant (B) strains before or after combined nitrogen starvation. A total of 1 µg of RNA was used in each experiment. Samples were collected at the indicated cycles of the exponential phase of the PCR. All experiments were repeated twice, with similar results obtained. Note that the samples were not collected during the same PCR cycles. (C) Representation of *patA* promoter region. The two putative conservative NtcA-binding boxes are boxed. The numbers are positions relative to the translational start site of *patA*.

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TABLE 1. Primers used in this study

Primer	Sequence (5'-3')	Feature
<i>patA</i> top	AATGAAAGATACAGAAAAGCAACC	Amplifies the <i>patA</i> promoter
<i>patA</i> bottom	TTGACATCATGAAACTATT	
<i>A</i> ₁ top	CCTGATTTTTGAAAGAATGTTTTCTAACGATCGG	Amplifies the <i>patA</i> promoter mutated in the -249 NtcA site
<i>A</i> ₁ bottom	TTGACATCATGAAACTATT	
<i>A</i> ₂ top	AATGAAAGATACAGAAAAGCAACC	Amplifies the <i>patA</i> promoter mutated in the -110 NtcA site
<i>A</i> ₂ bottom	CTGAGAATATATTTACCTAAGTTCAGCAATTGCTAGTTAAG	
<i>A</i> _{1/2} top	CCTGATTTTTGAAAGAATGTTTTCTAACGATCGG	Amplifies the <i>patA</i> promoter mutated in the two NtcA sites
<i>A</i> _{1/2} bottom	CTGAGAATATATTTACCTAAGTTCAGCAATTGCTAGTTAAG	RT-PCR: <i>mpB</i> transcript
<i>mpB</i> top	AGGGAGAGAGTAGGCGTTGG	
<i>mpB</i> bottom	GGTTTACCGAGCCAGTACCTCT	
<i>patA</i> top	GTAATAGTTGATAGGCGATCGCTCTGTT	RT-PCR: <i>patA</i> transcript
<i>patA</i> bottom	TTTCTGCAGGGCTGAACCTTTGGGAGTT	
<i>P</i> _{<i>patA</i>} top	GGAATTCCTTCAGGATTGGGGACAAGTGAATC	Amplifies the <i>P</i> _{<i>A</i>₁-<i>gfp</i>} fusion mutated in the -249 NtcA site
<i>P</i> _{<i>A</i>₁} bottom	CCGATCGTTAGAAAACATTCTTTCAAAAATCAGG	
<i>P</i> _{<i>A</i>₂} top	CTTAAGTAGCAATTGCTGAACCTTAGGTAAATATATTCTCAGA	Amplifies the <i>P</i> _{<i>A</i>₂-<i>gfp</i>} fusion mutated in the -110 NtcA site
<i>P</i> _{<i>A</i>₂} bottom	GGTACCTTCATTATTTGTAGAGCTCATCCA	

plified by PCR using the primers listed in Table 1. The GTA nucleotides of the putative NtcA-binding sites (GTAN8TAC) were substituted for ACG (*A*₁) and AGC (*A*₂). The DNA binding of NtcA to these promoter regions, in the presence of various concentrations of 2-OG, was assessed as previously described (3). At a concentration of 0.1 mM 2-OG, the interaction of NtcA with the *patA* promoter was greatly stimulated (Fig. 2). The mutation of the conserved NtcA-binding sites completely abolished the interaction of this regulator with the *patA* promoter region. Indeed, the shifted band disappeared when the mutated promoter region was used in the NtcA motility assay (Fig. 2, bottom).

Role of NtcA-binding sites in the expression of *patA* in vivo.

In order to better understand the mechanism by which NtcA activates the transcription of *patA*, we assessed the contributions of both of the NtcA-binding sites in the regulation of *patA* in vivo. Transcriptional fusions to the *gfp* reporter gene were constructed with the wild-type *patA* promoter or with its derivatives bearing mutations in either one or both of the NtcA-binding sites. The constructions were cloned

into the replicative plasmid pRL25c and introduced by conjugation into *Anabaena*. Recombinant clones were grown under combined nitrogen starvation. The transcription from the different *patA* promoters was analyzed by monitoring the fluorescence emitted by the cells. The *gfp* fusion with the wild-type *patA* promoter showed bright fluorescence in heterocysts (Fig. 3). The *P*_{*patA*-*gfp*} fusion was not expressed in the *ntcA* mutant (data not shown). The mutation of the two NtcA-binding sites abolished the expression of the *gfp* fusion (Fig. 3). The binding of NtcA to the *A*₂ site seems to be essential for *patA* activation, since its mutation was sufficient to abolish the expression of the *gfp* fusion (Fig. 3). On the other hand, *A*₁ mutation reduces only the level of *gfp* expression. All together, these data establish that NtcA binds directly to the two conserved sites and activates the expression of *patA* in *Anabaena* cells challenged with combined nitrogen starvation.

The expression levels of *hetR* and *ntcA* are mutually interdependent (7). It has been suggested that PatA might perceive a signal in developing cells and, in response, stimulates the

FIG. 2. NtcA interaction with the *patA* promoter. DNA-binding activity of NtcA toward the promoter region of *patA*, with various concentrations of 2-OG. The NtcA protein was purified as described in reference 6. DNA fragments used in electrophoretic mobility shift assays were obtained by PCR amplification carried out with the primers listed in Table 1. (Top) Assays carried out with the wild-type *patA* promoter region; (bottom) assays carried out with the *patA* promoter region bearing mutations in the two consensus NtcA sites. The arrow indicates DNA/protein complexes. All experiments were repeated twice, with similar results obtained.

FIG. 3. Fluorescence micrographs (left), light transmission micrographs (middle), and superposition of the two previously mentioned micrographs (right) of *Anabaena* sp. strain PCC 7120 bearing P_{patA} -gfp, P_{A1} -gfp, P_{A2} -gfp, or $P_{A1/2}$ -gfp. The P_{patA} -gfp fusions were generated from the pHB912/ P_{patA} -gfp plasmid (8). All the primers used to construct the gfp fusions are listed in Table 1. The images were obtained 24 h after combined nitrogen was removed from the culture. The arrows indicate heterocysts.

activity of HetR (1, 7). Our demonstration that *patA* expression is controlled by NtcA confirms that *patA* and *hetR* belong to the same regulatory circuit. The challenge ahead is to elucidate the mechanism whereby PatA perceives its signal and transmits it into HetR activation.

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