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Analysis of a Heterocyst-controlling Gene and Its Expression upon Nitrogen Starvation in a Cyanobacterium

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The filamentous cyanobacterium *Anabaena* is capable of both photosynthesis and nitrogen fixation which probably facilitated its incredible adaptation and proliferation in freshwater environments. A small gene, *patS*, was found to block nitrogen fixing cells from developing which resulted in death of *Anabaena* in the absence of combined nitrogen sources. We analyzed the DNA sequences in the vicinity of the *patS* gene by using a codon usage program and detected no codon bias other than the *patS* open reading frame. Three overlapping cosmids that contain the *patS* gene were identified, and the presence of other known heterocyst-controlling genes was examined. The *patS* expression in response to nitrogen starvation was analyzed at the level of transcription and translation by using Northern blot analyses and *lacZ*-reporter-gene fusion experiments, respectively. The *patS* expression increased rapidly (within 12 hours) upon the removal of combined nitrogen from the media.

Key words : heterocyst-controlling gene, expression pattern, nitrogen concentration, *Anabaena*, cyanobacteria

INTRODUCTION

Cyanobacteria are ancient organisms (Schopf, 1993) with an oxygen evolving photosynthetic apparatus that is similar to that of higher plant chloroplasts (Ho and Krogmann, 1982). Biological nitrogen fixation requires that nitrogenase be protected from oxygen and several mechanisms for meeting this requirement have evolved (Burris and Roberts, 1993). Some filamentous cyanobacteria capable of both oxygenic photosynthesis and nitrogen fixation exhibit a striking example of prokaryotic cellular differentiation to produce heterocysts, highly specialized cells that fix atmospheric nitrogen (Buikema and Haselkorn, 1993). Under nitrogen limiting conditions, *Anabaena* grows as a simple multicellular organism composed of two interdependent cell types: vegetative cells and heterocysts (Fig. 1A and B). Heterocyst formation probably represents one of the earliest examples of cellular specialization assuming that they were required during the time the Earth's atmosphere started to accumulate oxygen (Schopf and Walter, 1982). Approximately every tenth cell along the filament differentiates into a heterocyst forming a one dimensional developmental pattern. The regulation of heterocyst development involves both external cues and intercellular communication (Adams and Carr, 1981).

Heterocyst development requires global changes in gene expression with large numbers of genes, such as *rbcLS*, which is required for carbon fixation, being turned off and those involved in nitrogen fixation, e.g., *nifHDK*, being turned on (Haselkorn *et al*., 1983). Some genes, for example *glnA*, which codes for glutamine synthetase, are

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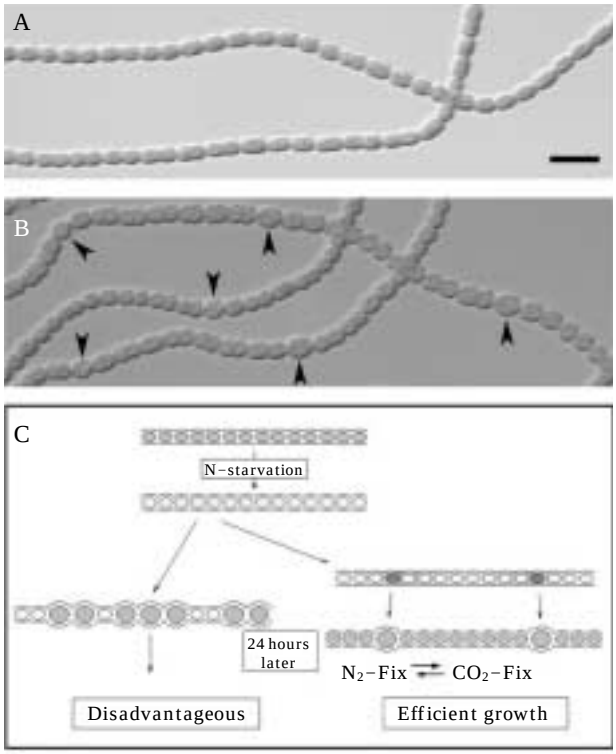


Fig. 1. Wild-type *Anabaena* filaments grown in the presence of enough nitrogen sources contain only photosynthetic vegetative cells (A). When combined nitrogen is limiting, one out of approximately ten vegetative cells along the filament differentiates into a nitrogen fixing specialized cell called heterocyst (arrowheads in B). The importance of heterocyst pattern is illustrated in (C). Scale bar, 10 μm.

expressed in both cell types (Tumer *et al.*, 1983). Changes in the protein patterns found in the two cell types show more differences than similarities and changes in gene expression measured at the mRNA level suggest that 15 to 25% of the genome is uniquely expressed in heterocysts (Lynn *et al.*, 1986). The heterocyst is terminally differentiated, lacks components of the oxygen-evolving photosystem II and, has a unique morphology and biochemistry that provides the anaerobic microenvironment necessary for nitrogen fixation (Adams and Carr, 1981). Heterocysts must obtain photosynthate from nearby vegetative cells and they export fixed nitrogen to the filament as glutamine. They produce a multilayered gas-impermeable cell wall and form specialized junctions with neighboring vegetative cells. The regular distribution and proper number of heterocysts are regulated to optimize the transportation of fixed nitrogen within a long filament to allow efficient growth (Fig. 1C).

Recent reviews discuss the genetic tools available for filamentous cyanobacteria and several of the recently identified genes that play a role in heterocyst differentiation and pattern formation (Buikema and Haselkorn, 1993). Among many genes that affect heterocyst development, *patS* was found to inhibit heterocyst formation causing the death of the cyanobacterium in the absence of combined nitrogen sources (Yoon and Golden, 1998). *patS* is a small 54-base-pair gene that can encode 17 amino acids. A chemically synthesized peptide corresponding to the last five amino acids of PatS blocked heterocyst formation completely in micro molar concentrations. PatS appears to control the nitrogen fixing heterocysts through intercellular signaling mechanisms.

In this report, we analyzed the *Anabaena* genome near the *patS* gene by using a codon usage program and by identifying overlapping cosmids. Also the expression pattern of the *patS* gene in response to changing nitrogen concentrations was analyzed at the transcriptional and translational levels.

MATERIALS AND METHODS

1) Strains and culture conditions

Anabaena sp. Strain PCC 7120 and derived strains were grown as previously described (Golden *et al.*, 1991). Essentially, cultures were grown in 100-mL BG-11 liquid media with shaking at 30°C with 50 to 80 μE m^{-2} s^{-1} of illumination or on plates of BG-11 containing 1.5% agar at 30°C with 100 to 120 μE m^{-2} s^{-1} of illumination. Heterocysts were induced by growth in liquid or solid BG-11 medium lacking sodium nitrate (BG_0). *Escherichia coli* strains DH5-α MCR and DH10B (Gibco-BRL) were used for plasmid maintenance and were grown in LB liquid or on LB agar medium (Lennox L) or 0.5 × TB medium, a modified form of Terrific Broth (Tartof and Hobbs, 1987). Media were supplemented with appropriate antibiotics at standard concentrations (Ausubel, 1994).

2) DNA manipulations

DNA manipulations and recombinant DNA techniques were performed by standard procedures (Ausubel, 1994). Restriction endonucleases

and other DNA-modifying enzymes were used according to the manufacturer's recommendations or standard protocols.

3) Southern analysis

The genomic and cosmid DNA was digested by restriction enzymes *Cla*I and *Hind*III, separated on 0.7% agarose gels, and transferred to MagnaCharge membranes (MSI) with 50 mM NaOH, 1 M NaCl. DNA probes were labeled using a random primer kit (Boehringer Mannheim), and Southern hybridizations were performed as described (Golden *et al.*, 1991). A *Bam*HI-*Cla*I fragment containing *patS* was used as the probe.

4) Northern analysis

RNA from differentiating filaments was prepared from frozen samples of a large-scale heterocyst induction of wild-type *Anabaena* PCC 7120 by centrifugation through 5.7 M CsCl, as previously described (Wei *et al.*, 1994). 20 μg RNA samples were denatured with formaldehyde, separated by electrophoresis on a 1.2% formaldehyde-agarose gel, and transferred to a MagnaCharge membrane (MSI) with 10X SSPE. The blot was then hybridized with a strand-specific probe, generated by using Maxiscript (Ambion), at 55°C in a solution containing 50% formamide and washed at 70°C in 0.5×SSPE and 0.1% SDS. The resulting autoradiogram was scanned and analyzed by a density measuring program. The density of *patS* transcripts (signal) was compared with the cross-hybridized 16S RNA transcripts (noise) in each lane. The result of the calculated S/N ratio of the *patS* signal was plotted.

5) *pat*S-*lacZ* fusion construction and β-galactosi-dase assay

To construct in-frame and out-of-frame *patS-lacZ* translational fusions, DNA fragments containing the 5′ leader and the coding sequences of *patS* were generated by PCR with PFU polymerase (Stratagene). The forward primer was 5′-CGCTCTAGAACTAGTGGATC-3′ and the two different reverse primers each contained an engineered *Sma*I site (underlined): 5′-CGCCCGGGTCTACCACTACCGCGCTC-3′ and 5′-CGCCCGGGTCTATCTACCACTACCGC-3′ for in-frame and out-of-frame constructions, respectively. After digestion with *Eco*RV-*Sma*I, the fragments were ligated to the promoterless *lacZ* gene in pMC1871 (Shapira *et al.*, 1983) at the *Sma*I site. The fusions were isolated by *Sal*I digestion and cloned into shuttle vector pAM504. In-frame (pAM1899) and out-of-frame (pAM1860) *patS-lacZ* translational fusions were conjugated into *Anabaena* PCC 7120. The nucleotide sequence of the insert was confirmed for both clones. Several independent exconjugants were cultured in liquid BG-11 medium and harvested at OD750 = 0.3 for β-galactosidase assays. Cells were lysed as previously described (Schaefer and Golden, 1989) except that the filaments were frozen at −85°C before processing. The concentration of protein was determined by the method of Lowry, and β-galactosidase activity was measured colorimetrically as previously described (Schaefer and Golden, 1989) and expressed as specific activity (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein).

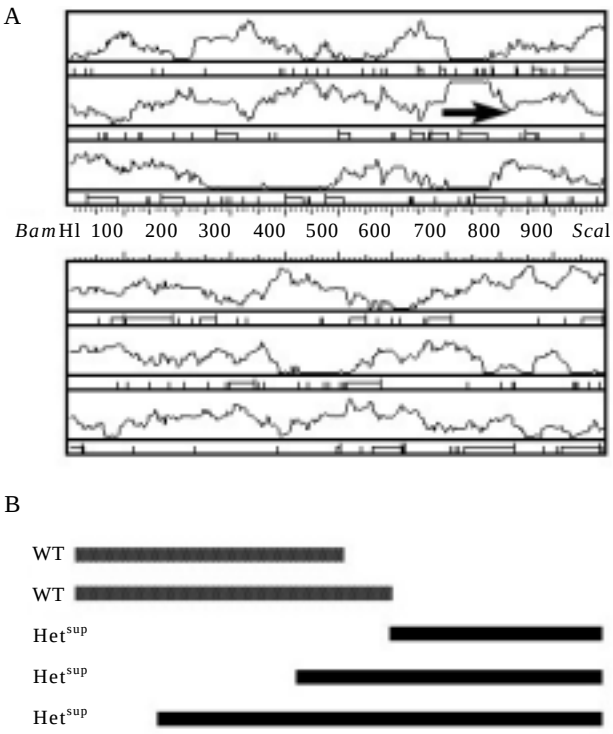


Fig. 2. The 950-bp *Bam*HI-*Sca*I DNA fragment that showed heterocyst inhibition activity was analyzed by Graphical Codon Usage Analysis 1.2.1 for other possible heterocyst-controlling DNA elements. All 6 frames showed no significant codon bias other than the *patS* ORF (indicated as an arrow in the second reading frame of the panel A). Subcloning experiments indicates *patS* is the only gene responsible for the heterocyst suppression (Het^{sup}) phenotype (B).

RESULTS

1. Analysis of a heterocyst controlling DNA region by a codon−usage program and overexpression experiments

A cosmid library containing random 30− to 35−kb fragments of the *Anabaena* PCC 7120 genome was constructed in the conjugal shuttle vector pDUCA7M and a cosmid 8E11 was found to suppress heterocyst development leading to the formation of yellow and nongrowing colonies on nitrogen−free medium. Subcloning experiments of 8E11 were used to identify a 3.3−kb *Bam*HI−*Cla*I fragment that produced the heterocyst suppression (Het^{sup}) phenotype (Yoon and Golden, 1998). The DNA sequence was determined for this region. Further subcloning showed that a 950−bp *Bam*HI−*Sca*I fragment was responsible for the Het^{sup} phenotype. Within the *Bam*HI−*Sca*I fragment, a small gene *patS* was known to block heterocyst formation (Yoon and Golden, 1998). To further analyze the *Bam*HI−*Sca*I fragment for other possible heterocyst−controlling DNA elements, a codon usage program (Graphical Codon Usage Analysis 1.2.1 and codon usage table for cyanobacterium) was used to scan the sequences of the fragment in all 6 frames. The only open reading frame (ORF) that showed significant bias in its codon usage was the *patS* ORF (Fig. 2A). Various DNA fragments covering the 950−bp *Bam*HI−*Sca*I region were subcloned and overexpressed to test if other DNA elements in that region might affect heterocyst differentiation. No altered effects on heterocyst development were detected upon conjugation into *Anabaena* (Fig. 2B). We concluded that *patS* is the only gene that controls heterocyst formation in that 950−bp *Bam*HI−*Sca*I fragment. However, the possibility of a criptic cis−regulating element in the 950−bp region that affect heterocyst development still remains.

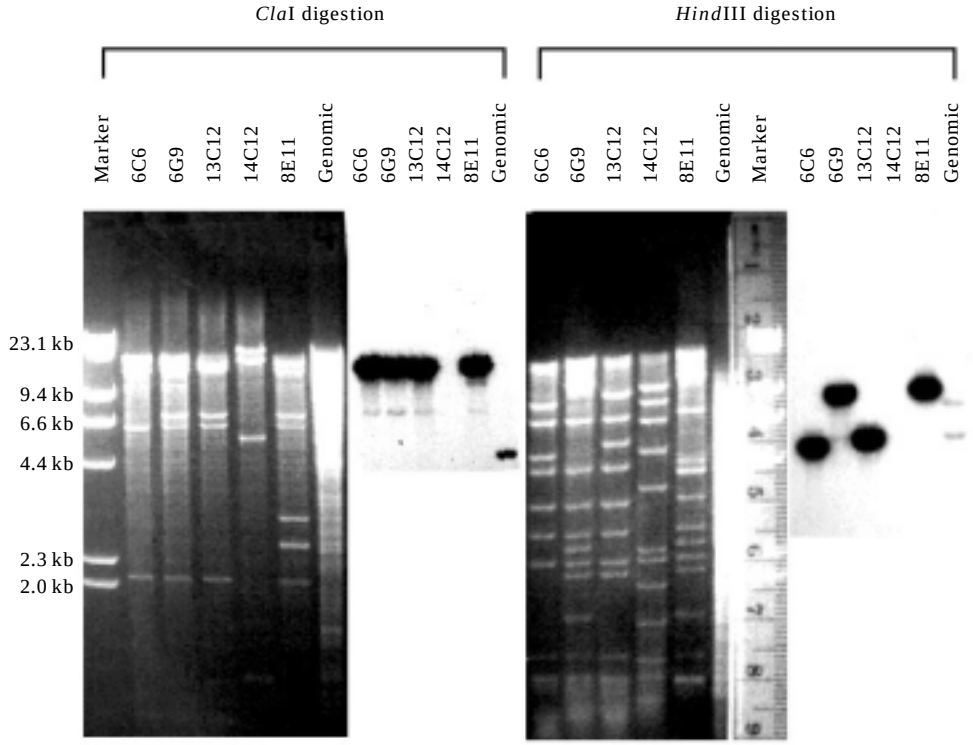


Fig. 3. Five cosmid and *Anabaena* genomic DNA was digested with restriction enzymes *Cla*I and *Hind*III, and separated on 0.7% agarose gels (left panels with dark background). Southern blot analysis was performed using a probe containing the *patS* gene (right panels with white background). Four cosmids including 8E11 showed strong hybridization with the *patS* probe (except 14C12). Genomic DNA lanes show weak and different size bands due to the low copy number (when compared with the cosmids) and different restriction sites than the vector containing cosmids, respectively.

2. Identification of overlapping cosmids

The 8E11 cosmid was mapped to the same region as the heterocyst-regulating genes, *hetR*, *glnA* and *petA* genes, between approximately 2.7 and 3.0 megabases on the *Anabaena* PCC 7120 chromosome (Kuritz *et al*., 1993). An 8E11 probe hybridized to *Bln*I (*Abr*II) fragment D, *Sal*I fragment H, and *Pst*I fragment C. Because *hetR* is known to regulate heterocyst development (Buikema and Haselkorn, 1991) and *glnA* is involved in nitrogen metabolism (Haselkorn *et al*., 1983), we set out to determine if they were on or closely linked to the DNA carried on cosmid 8E11. A 3.3 kb *Bam*HI-*Cla*I fragment from pAM1023 was used as a probe to identify overlapping cosmids in the pDUCA7M library and three clones, 6C6, 6G9, and 13C12, were identified (Fig. 3). 14C12 was initially found to hybridize to the 3.3 kb *Bam*HI-*Cla*I probe, but further analysis showed that 14C12 did not hybridize with the probe (Fig. 3). The *hetR* and *glnA* probes did not hybridize with 8E11 or the three overlapping cosmids. The 3.3-kb *Bam*HI-*Cla*I fragments from the 3 overlapping cosmids were isolated and cloned into shuttle vectors. Those fragments produced the same Het^{sup} phenotype as 8E11, indicating that the dominant Het^{sup} phenotype is a property of wild-type sequences.

3. Expression pattern in response to nitrogen starvation

The expression of *patS* during heterocyst differentiation was examined by Northern blot analysis (Fig. 4). The *patS* transcript was detected in vegetative cells grown on nitrate and also seen at 6 and 12 hours after heterocyst induction. The *patS* messages appeared as a smear between 0.5 and 1 kb in vegetative-cell RNA, and between 0.2 to 1 kb in the RNA from induced filaments. The size distribution of messages is significantly larger than the 54-bp *patS* ORF. The *patS* transcripts may have a long leader region, or they may terminate far downstream of the ORF.

The same RNA samples have previously been analyzed for the expression of *nifHDK*, *glnA*, and *ntcA* (Wei *et al*., 1994). The *nifHDK* message was absent from vegetative cells and at 6 and 12 hours after heterocyst induction. An abundant *nifH* 1.1 kb message was detected at 18 hours and later after nitrogen step-down (data not shown). In this experiment, mature heterocysts

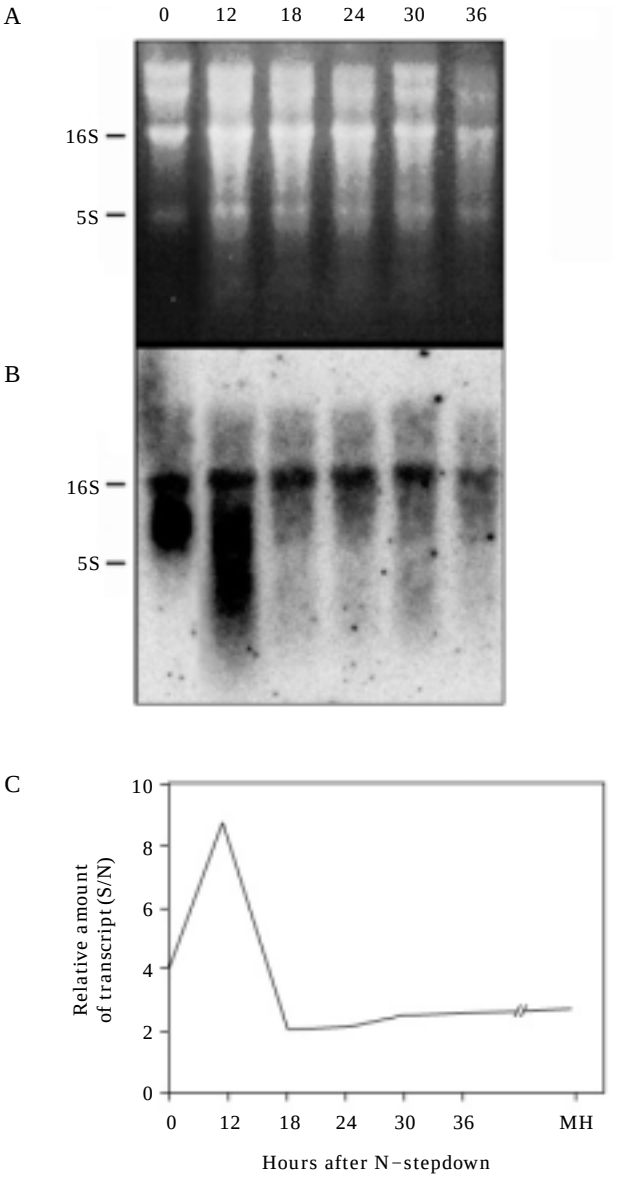
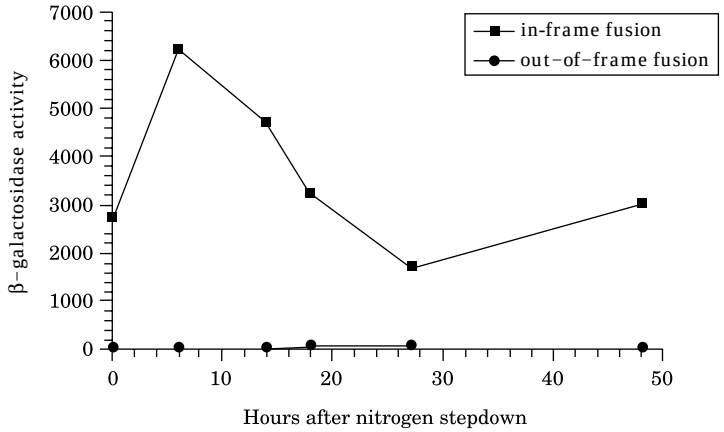


Fig. 4. Northern blot analysis of *patS* transcripts upon removal of nitrogen sources. Total RNA was isolated from vegetative cells grown on nitrate (0 hr) and from filaments harvested at 12, 18, 24, 30, and 36 hours after nitrogen step-down. A strand-specific *patS* probe was used. An EtBr stained agarose gel picture (A, as a loading control) and a Northern blot that was hybridized to *patS* probe (B) are shown. The positions of 16S and 5S ribosomal RNA are indicated as size references. The resulting autoradiogram was analyzed by a density scanning program and the density of *patS* transcripts (signal) was compared with the cross-hybridized 16S RNA transcripts (noise) in each lane. The result of the calculated S/N ratio of the *patS* signal was plotted (C). MH indicates RNA from isolated mature heterocysts.



in−frame translational fusion

AAAAGTAATTCACCGATTTTAAGATTATGAAGGCAATTATGTTAGTGAATTTCTGTGATGAGCGCGGTAGTGGTAGACCC
M K A I M L V N F C D E R G S G R P

out−of−frame translational fusion

AAAAGTAATTCACCGATTTTAAGATTATGAAGGCAATTATGTTAGTGAATTTCTGTGATGAGCGCGGTAGTGGTAGATAGACCC
M K A I M L V N F C D E R G S G R *
M S A V V V D R P

Fig. 5. Expression pattern of the *patS−lacZ* translational fusion in response to nitrogen starvation. The β−galactosidase specific activity of *Anabaena* exconjugants containing in−frame and out−of−frame *patS−lacZ* fusions was measured at indicated times after nitrogen step−down. One representative result of three independent experiments is shown. Sequences of in−frame and out−of−frame *patS−lacZ* translational fusions are shown.

began to be observed approximately 24 hours after induction.

4. *patS−lacZ* expression increases after nitrogen step−down

The β−galactosidase specific activity in *Anabaena* PCC 7120 strains containing in−frame and out−of−frame *patS−lacZ* fusions was determined after nitrogen step−down (Fig. 5). Basal levels of β−galactosidase activity were detected in vegetative cells containing the *patS−lacZ* in−frame construction (pAM1899), and the specific activity increased 2 to 3 fold early after heterocyst induction. The specific activity gradually decreased at later times, reaching its lowest level at 24 hours, which coincides with the time of heterocyst maturation. The strain containing the *patS−lacZ* out−of−frame construction (pAM1860) did not produce any significant β−galactosidase activity (Fig. 5).

DISCUSSION

The product of the small gene *patS* blocks heterocyst formation when it is overexpressed. Addition of a pentapeptide corresponding to the last five COOH residues of *patS* to cultures at 1 μM also inhibited heterocyst differentiation, suggesting that *PatS* may be a diffusible inhibitor. The *patS*−deletion mutant displayed long chains of contiguous heterocysts, which could be the result of a defect in communication between adjacent differentiating cells. These findings support our view that the *PatS* peptide is normally expressed by proheterocysts and signals the neighboring cells to stay in the vegetative state. In this report we showed, by Northern RNA analysis and *patS−lacZ* fusion analysis, respectively, that both transcription and translation of *patS* increase early after the onset of differentiation. We tested whether the *patS* ORF was actually translated by making a translational fusion with *lacZ*. As a control, an out−of−frame *patS−lacZ* construct was made. *Anabaena* exconjugants containing the in−frame *patS−lacZ* fusion produced more than 2000 units of β−galactosidase specific activity. The control strain containing the out−of−frame construct showed only a background level of β−

galactosidase specific activity (0 to 20 units). This result indicates that the *patS* ORF is translated in *Anabaena* PCC 7120. Also in other experiments, the *patS* increase was shown to be specific to cells destined to become heterocysts by analyzing the expression pattern of a *patS-gfp* fusion (Yoon and Golden, 2001). At later times during differentiation, *patS-gfp* expression was detected exclusively in proheterocysts, a result which corresponds well to the result that *patS*-deletion strain AMC451 can be complemented *patS* expression driven by the heterocyst-specific *hepA* promoter (Yoon and Golden, 1998).

PatS may also play an important role in maintaining the heterocyst pattern after heterocysts mature and supply nitrogen-fixation products to the filament. At about the same time that differentiation is completed and heterocysts start fixing nitrogen, *patS* expression decreases in filaments, as shown by Northern analysis and the β-galactosidase activity supported by a *patS-lacZ* reporter fusion. It is thought that the nitrogen-fixation product contributes to the maintenance of pattern, since cells adjacent to heterocysts would no longer be nitrogen limited. However, as the vegetative cells resume growth and reproduce by binary fission, the distance between two heterocysts increases, resulting in cells midway between two heterocysts that become deficient in nitrogen supply. These nitrogen-deprived cells will initiate differentiation, and we found that *patS-gfp* expression increased in these cells (Yoon and Golden, 2001). The function of *patS* also seems critical in this situation to prevent adjacent cells from simultaneously differentiating by allowing one cell in the cluster to inhibit differentiation of nearby cells.

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＜**국문적요**＞

남조류의 이형세포 조절 유전자와 질소량에 따른 유전자 발현의 분석

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선형의 남조류 아나베나는 광합성과 질소고정 능이 있으며, 이는 아마도 이 남조류가 각종 육수 환경에 잘 적응하는데 큰 역할을 했다고 볼 수 있다. 작은 *patS*라는 유전자는 질소 고정세포의 형성을 막으며 결과적으로 질소원이 부족한 환경에서 아나베나의 죽음을 가져온다. 본 연구는 *patS* 유전자 주변의 DNA 염기 서열을 분석하여 codon 활용도를 알아 본 결과 *patS*를 제외한 다른 유전자가 존재하지 않음을 밝혔다. *patS*를 포함하는 세 개의 겹치는 cosmid를 찾아서 기존의 알려진 이형세포 발달 유전자를 탐색하였으나 나타나지 않았다. 질소원의 결핍에 반응하는 *patS* 유전자의 발현을 Northern blot 분석과 *lacZ* reporter 유전자 합성 실험을 통하여 각각 전사와 번역의 단계에서 알아보았다. *patS* 유전자의 발현은 배지로부터 질소원이 제거된 후 12시간 내에 급격히 증가하는 것으로 나타났다.