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Epistatic Relationships of Two Regulatory Factors During Heterocyst Development

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The filamentous cyanobacterium *Anabaena* sp. Strain PCC 7120 produces a developmental pattern of single heterocysts separated by approximately 10 vegetative cells. Heterocysts differentiate from vegetative cells and are specialized for nitrogen fixation. The *patS* gene, which encodes a small peptide that inhibits heterocyst differentiation, is expressed in proheterocysts and plays a critical role in establishing the heterocyst pattern. Another key regulator of heterocyst development is the *hetR* gene. *hetR* mutants fail to produce heterocysts and extra copies of *hetR* on a plasmid cause a multiple contiguous heterocyst phenotype. To elucidate the relationship between these two counter acting factors in the genetic regulatory pathway during heterocyst differentiation, the expression patterns of a *patS-gfp* and a *hetR-gfp* fusion were examined in a *patS* deletion and a *hetR* deletion strain. The results, in combination with the result from a *hetR* and *patS* double deletion strain, suggest *patS* and *hetR* are mutually antagonistic and the balance between these two factors in two different cell types (heterocysts and vegetative cells) may be critical during the decision making process on their cell fates.

Key Words: cellular differentiation, developmental genes, diffusible signal, epistatic relationship, mutual antagonistic regulation

INTRODUCTION

Cyanobacteria, previously known as blue-green algae, are a large and diverse group of photosynthetic bacteria representing one of the major phylogenetic lines of eubacteria (Carr and Whitton 1982). Being widely distributed in nature in terrestrial, freshwater, and marine habitats, cyanobacteria are also often dominant photosynthetic organisms in extreme environments, including hot springs, saline lakes, and desert soils. Blooms of cyanobacteria may develop in fresh water lakes in times when they are rich in nutrients. Some filamentous cyanobacteria exhibit an interesting example of cellular differentiation to produce heterocysts, highly specialized cells that fix atmospheric nitrogen. The differentiation of a photosynthetic vegetative cell into a nitrogen-fixing heterocyst is regulated by the availability of combined nitrogen and intercellular communication, and results in a semi-regular pattern of heterocysts along the vegetative cell filament.

Anabaena sp. strain PCC 7120 (*Anabaena* PCC 7120) grows as filaments of vegetative cells in media containing combined nitrogen. Vegetative cells carry out oxygenic photosynthesis similar to that of algae and higher plants (Ho and Krogmann 1982). When *Anabaena* PCC 7120 is grown in the absence of combined nitrogen, one out of approximately ten vegetative cells differentiates into a heterocyst. The terminally differentiated heterocysts are highly specialized cells in which nitrogen fixation occurs (Fay 1992). The oxygen-sensitive nitrogenase, the main enzyme complex for catalyzing the reduction of dinitrogen to ammonia, is present only in heterocysts (Bergman *et al.* 1986). The thick and non-permeable envelope of the heterocyst provides anaerobic conditions for nitrogenase in aerobically growing filaments (Walsby 1985). The differentiation of a vegetative cell into a heterocyst takes approximately 24 hours and culminates with the expression of the *nif* genes and the reduction of nitrogen to ammonia. Heterocyst differentiation involves a

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large number of structural, biochemical, and genetic changes (Wolk *et al.* 1994).

Cyanobacterial heterocyst differentiation involves the processing of environmental and intercellular signals to produce a developmental pattern of highly specialized terminally differentiated cells. The process of heterocyst differentiation is complex and involves the coordinated regulation of large numbers of genes. This study is focused on identifying genes that are required for development and understanding their role in the process of heterocyst differentiation.

A *hetR* mutant strain fails to produce heterocysts in the absence of combined nitrogen. Extra copies of the *hetR* gene on a plasmid lead to supernumerary heterocysts in wild-type cells. The *hetR* is expressed at a low constitutive level in vegetative cells, and the level of *hetR* mRNA increases at least 20-fold within 6 hours of nitrogen stepdown. Studies with a *hetR-luxAB* fusion indicate that *hetR* is induced only in cells that are designated to differentiate into heterocysts and that a functional *hetR* gene is required for its own increased expression during development. Expression of another developmentally regulated gene, *hepA*, whose product is involved in heterocyst envelope formation, requires intact *hetR*. The *hetR* gene product may play a central role in heterocyst development (Buikema and Haselkorn 1991a; Buikema and Haselkorn 1991b).

The simple, one-dimensional developmental pattern spatially separates two incompatible processes into different cell types: oxygen-evolving photosynthesis in vegetative cells and oxygen-sensitive nitrogen fixation in heterocysts. These two cell types cooperate by exchange of their products: carbohydrates and fixed nitrogen, respectively. It is assumed that multiple internal and external signals must be integrated within a regulatory network that controls cell identity and pattern formation. The *patS* gene was identified during genetic experiments Bauer *et al.* (1997) designed to find genes involved in heterocyst differentiation. We have found that a small gene, *patS*, is crucial for the formation and maintenance of a normal pattern of single heterocysts separated by at least 7 to 10 vegetative cells. The *patS* product appears to be an inhibitory peptide that functions in cell-cell signaling. To place *patS* into the developmental regulation pathway, its epistatic relationships with other known genes involved in heterocyst differentiation is required. For example, an *Anabaena* strain containing both extra copies of *hetR* (heterocyst stimulation) and *patS* (heterocyst inhibition) was generated to determine their epistatic interactions (Christine *et al.* 2006; Kim *et al.* 2008). Double knock-out strains, such as *patS*$^{-}$ (heterocyst stimulation) and *hetR*$^{-}$ (Het^{-}), was also generated (Christine *et al.* 2006; Kim *et al.* 2008). In this study, we examined the interaction of *patS* and *hetR* by using *gfp* reporter constructs.

MATERIALS AND METHODS

Cultures and molecular techniques

Anabaena PCC 7120 and derived strains were grown as previously described (Golden *et al.* 1991). BG-11 medium contains $NaNO_3$ (17.6 mM) as the nitrogen source, and BG-11_0 lacks a source of combined nitrogen. Subclones were transferred into *Anabaena* PCC 7120 by conjugation (Black *et al.* 1993). *Escherichia* coli strains were maintained in LB liquid or on LB agar medium (Lennox L). For plasmid preparation, strains were grown in 0.5 x TB liquid medium as described previously (Tartof and Hobbs 1987). Media were supplemented with appropriate antibiotics according to standard protocols (Ausubel *et al.* 1994). *E. coli* strain DH10B was used for plasmid maintenance. Cloning and molecular techniques were performed as previously described (Ausubel *et al.* 1994).

Plasmid constructions

The *patS-gfp* fusion construct was made by digesting pAM1035 with *Bam*HI and *Tha*I to isolate a fragment containing part of *patS* and an upstream region. Then the fragment was fused with a plasmid containing the pKEN2-GFPmut2 (Cormack *et al.* 1996), which was digested with *Xba*I, blunted with the Klenow fragment of DNA polymerase, and digested again with *Bam*HI. The resulting plasmid was then digested with *Hin*dIII, filled in by the Klenow enzyme, and digested with *Sac*I to release *patS-gfp* on a fragment that was then ligated into a shuttle vector digested with *Sac*I and *Sma*I. The *patS-gfp* fusion construct was finally confirmed by restriction digestion and DNA sequencing.

The *hetR-gfp* plasmid in which *gfp* was expressed from the *hetR* promoter (Black *et al.* 1993) was made by digesting the pKEN2-GFPmut2 with *Hin*dIII, filling in with Klenow enzyme, and digesting again with *Bam*HI to isolate the *gfp* gene. This fragment was then ligated into a *hetR* promoter that had been digested with *Sac*I, blunted with T4 polymerase, and digested with *Bam*HI. These steps place the *gfp* gene at the 3′ end of the *hetR* promoter fragment in a shuttle vector. The resulting plasmid was confirmed by restriction digestion and DNA sequencing.

Inactivation of the *patS* gene in the chromosome of *Anabaena* PCC 7120

The *patS* gene was deleted from the chromosome by obtaining double recombinants with suicide plasmid pAM1702 as previously described (Wei *et al.* 1994). Plasmid pAM1702 contains a 570-bp *Bam*HI-*Eco*RV fragment (leftward flanking sequence), an Ω (omega) cassette (conferring spectinomycin and streptomycin resistance), and an 895-bp *Sca*I-*Pac*I fragment (rightward flanking sequence) in the *Nru*I site of the *sacB*-containing suicide vector pRL278 (Black *et al.* 1993). The Ω cassette replaces a 381-bp *Eco*RV-*Sca*I fragment that contains the entire *patS* gene. Four independent isolates with identical phenotypes were obtained after selection on media containing 5% sucrose, spectinomycin (2 μg ml^{-1}), and streptomycin (2 μg ml^{-1}). The insertion of the deletion into the chromosome was confirmed by Southern analysis.

Fluorescence photographs

Fluorescence photographs were taken through filter sets of FITC specific illumination (484 ± 8 nm) and GFP specific emission (518 ± 13 nm) mounted on a Zeiss Axioplan II microscope. Hamamatsu 3CCD camera C5810 (1/2 inch interline CCD x 3) was used which was attached to the microscope via a HR coupler (Diagnostic Instruments, Inc.).

RESULTS AND DISCUSSION

GFP fluorescence assay of the *patS-gfp* fusion constructs in various mutant backgrounds

To analyze the temporal and spatial pattern of *patS* expression in *patS* and *hetR* mutant strains, a reporter gene encoding an optimized mutant version of green fluorescent protein (GFPmut2) of the jellyfish *Aequorea victoria* was used (Cormack *et al.* 1996). The *patS-gfp* transcriptional fusion on a low copy number plasmid was conjugated into wild-type *Anabaena* PCC 7120. Low-level expression of the *patS-gfp* fusion all along the filament was observed in nitrate-grown cultures (Fig. 1). Early after the onset of heterocyst differentiation (at 6 to 8 hour), GFP fluorescence appeared in some individual cells in a pattern that resembles the pattern of heterocysts. However, lower levels of GFP fluorescence were also observed in two adjacent cells, groups of cells, and cells with short stretches (3 to 6 cells) of non-fluorescing cells between them. Morphological differentiation of proheterocysts with a distinct pattern was distinguishable under these induction conditions at about 12 hours after nitrogen deprivation, and proheterocysts were the most brightly fluorescent cells at this time. At 16 to 18 hours after induction, the GFP fluorescence was seen almost exclusively in proheterocysts.

Although the GFP fluorescence was retained in mature heterocysts at 24 hours, the fluorescence from heterocysts at later times was much reduced, possibly because of the reducing environment in heterocysts (Wolk *et al.* 1994). After 24 hours, the filaments resume growth, and vegetative cells between heterocysts multiply once heterocysts start supplying fixed nitrogen. Beginning at 36 hours after nitrogen stepdown, GFP fluorescence was prominent in the cells midway between two heterocysts. Single fluorescing cells as well as two adjacent or short chains of fluorescing cells were visible. The fluorescence resolved mostly to single cells as the second round of heterocyst formation progressed.

The *patS-gfp* fusion construct in a *patS* mutant strain showed fluorescence in all the developing heterocysts (Fig. 1; Table 1). As reported previously, the *patS* deletion strain forms multiple contiguous heterocysts in nitrogen-deprived medium (Yoon and Golden 1998). The *hetR* mutant strain did not form heterocysts, and no indication of GFP fluorescence was observed in the *hetR* mutant strain (Table 1). Also in a *patS* and *hetR* double knock-out strain, no GFP fluorescence was detected. The *patS-gfp* reporter did not show fluorescence unless *hetR* is retained and proper heterocysts developed, which indicates that *patS* is under the positive control of *hetR*.

***hetR-gfp* fusion constructs in various mutant backgrounds**

The *hetR-gfp* reporter constructs in wild-type *Anabaena* PCC 7120 showed similar results as the case of *patS-gfp*. In *patS* deletion strain, the *hetR-gfp* fusion construct displayed strong fluorescence in all the developing heterocysts (Fig. 2). The temporal regulation of the *hetR-gfp* expression was also very similar to that of the *patS-gfp*. No fluorescence was detected in a *hetR* mutant or in a *patS* and *hetR* double mutant strain (Table 1). These results indicate that *hetR* is under the positive control of *hetR* itself. Also we can postulate that *patS* negatively regulates *hetR* since *hetR* was strongly expressed in the *patS* deletion strain. The mutually antagonistic relationship of the *patS* and *hetR* may produce a balance between the positive (*hetR*) and negative (*patS*) regulators which plays as a switch to turn on or off the heterocyst development program in different cell types. Detailed model

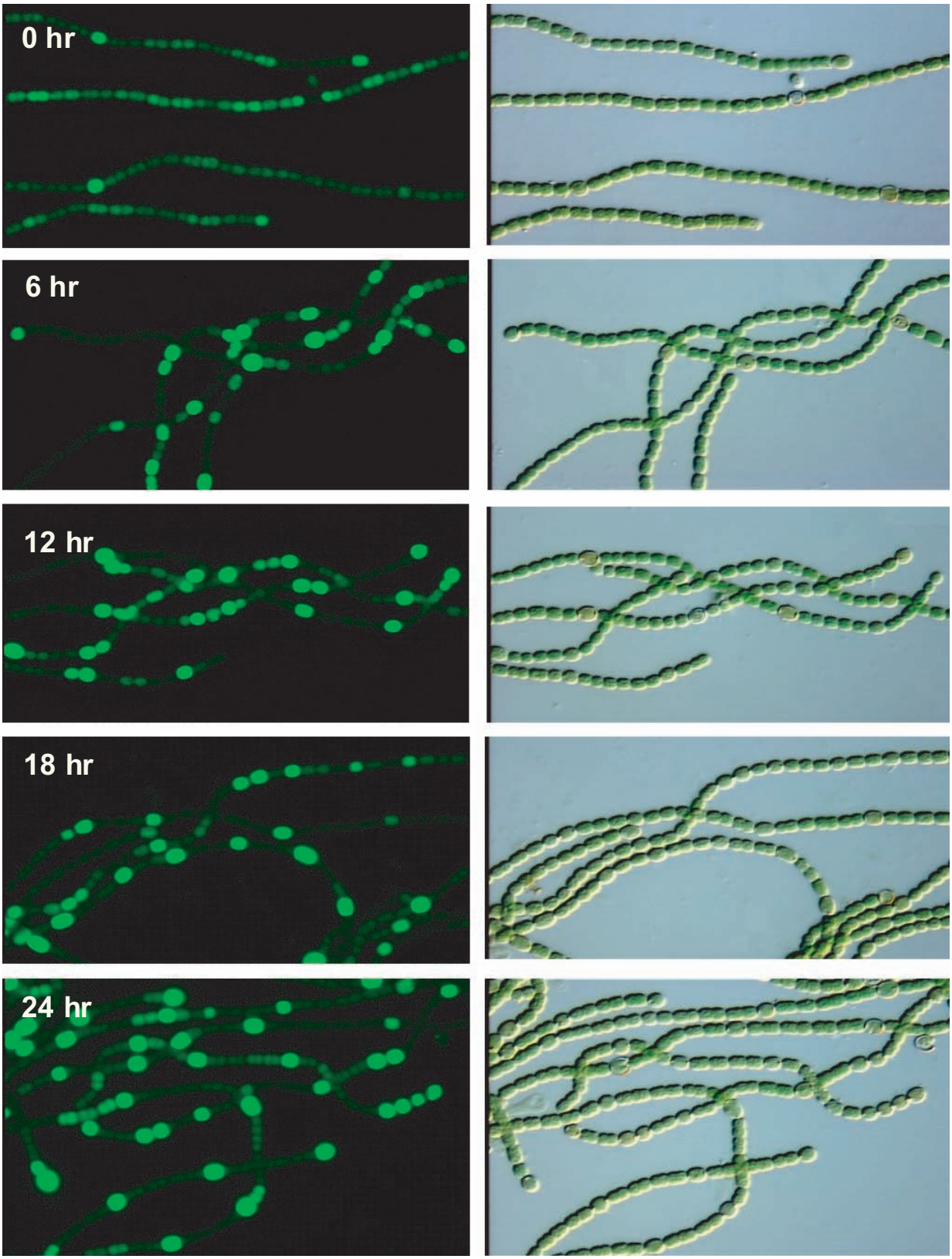

Fig. 1. Temporal and spatial expressions of a *patS-gfp* reporter in a *patS* deletion strain. GFP fluorescence (left panels) and the corresponding DIC photomicrographs (right panels). GFP fluorescence is shown in all the developing heterocysts.

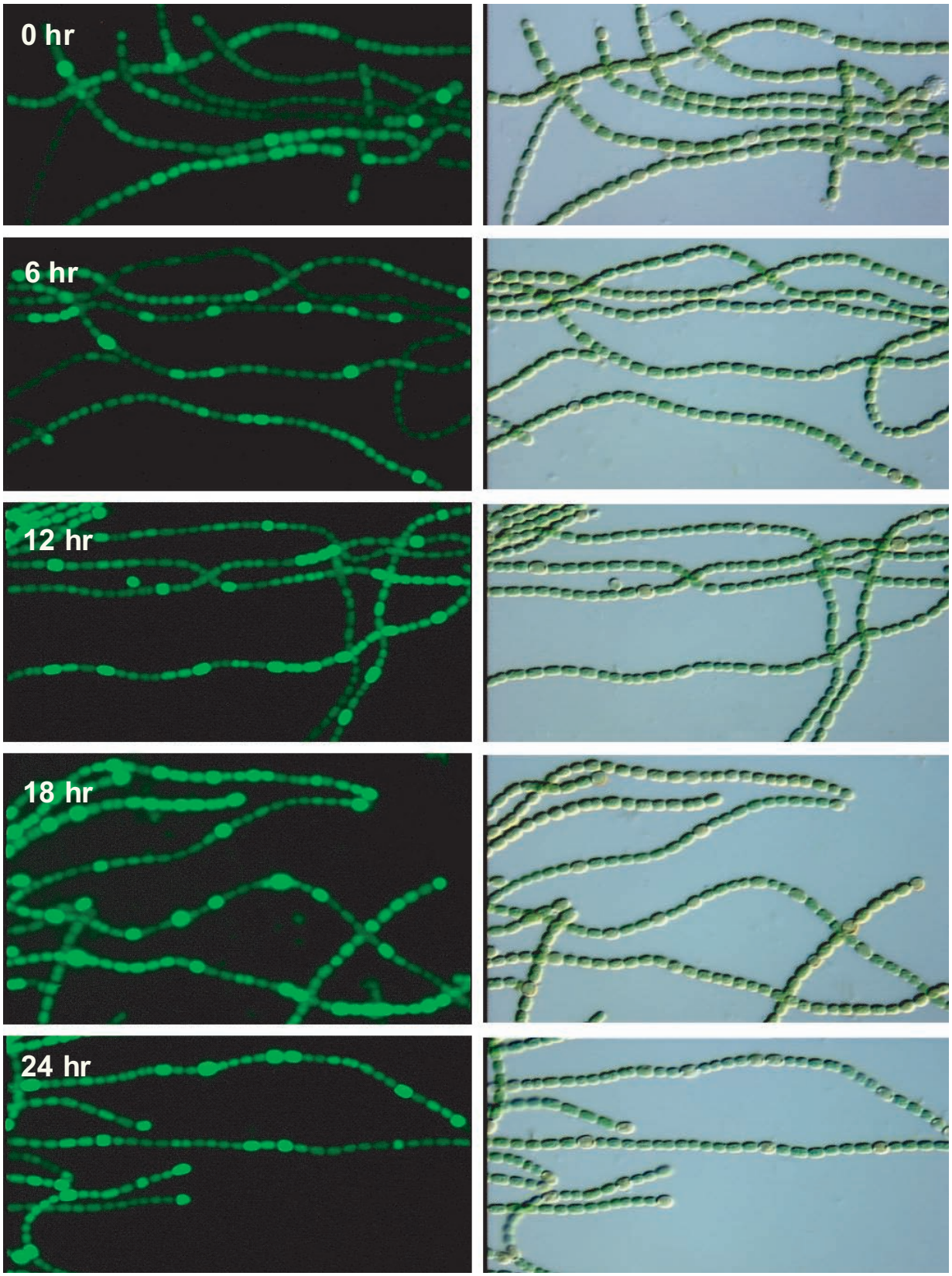

Fig. 2. Temporal and spatial expressions of a *hetR-gfp* reporter in a *patS* deletion strain. GFP fluorescence (left panels) and the corresponding DIC photomicrographs (right panels). GFP fluorescence is shown in all the developing heterocysts.

Table 1. GFP fluorescence and heterocyst formation in various mutant strains of *Anabaena* PCC 7120

Genetic background	*gfp* reporter fused to	Hets	fluorescence
WT	*patS* full length	-	-
WT	*patS* (truncated)	+	+
WT	*hetR* (truncated)	+	+
patS$^-$	*patS* full length	-	-
patS$^-$	*patS* (truncated)	+++	Fig. 1
patS$^-$	*hetR* (truncated)	+++	Fig. 2
hetR$^-$	*patS* full length	-	-
hetR$^-$	*patS* (truncated)	-	-
hetR$^-$	*hetR* (truncated)	-	-
patS$^-$, *hetR*$^-$	*patS* full length	-	-
patS$^-$, *hetR*$^-$	*patS* (truncated)	-	-
patS$^-$, *hetR*$^-$	*hetR* (truncated)	-	-

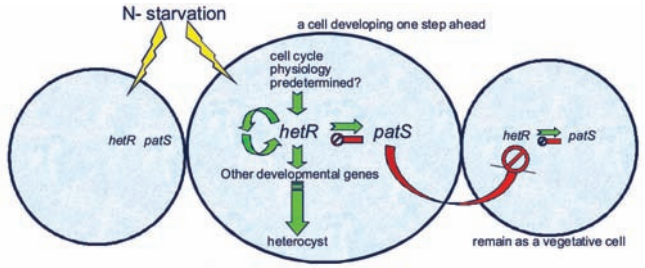

Fig. 3. A model of the *patS* and *hetR* regulatory relationships during the heterocyst development. Nitrogen starvation signal triggers *hetR* expression which is autoregulatory and amplifies itself. The *patS* gene is one of the positively regulated downstream elements by the *hetR* product. The *patS* product diffuses to neighboring cells to inhibit the master regulator *hetR* which results in halting the remaining developmental program.

regarding the balance of the regulators are discussed in next section and Fig. 3.

***patS* is downstream of *hetR* in the regulatory pathway and the balance determines heterocyst differentiation**

PatS was shown to be a diffusible inhibitor that regulates heterocyst pattern formation (Yoon and Golden 2001). The product of the small gene *patS* suppresses heterocyst development 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. This multiple-heterocyst phenotype was complemented by expression of *patS* from an early heterocyst-specific promoter. 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. Reporter gene assay indicates that *patS* negatively and *hetR* positively regulate both *patS* and *hetR* expressions. A model of the *patS* and *hetR* regulatory relationships and their role in heterocyst pattern formation was depicted in Fig. 3. *hetR* is autoregulatory and HetR is confined within developing heterocysts. PatS is a small peptide that diffuses to neighboring cells. In a cell that began heterocyst development ahead of other cells by various physiological reasons (see Fig. 3), *hetR* expression increases and the expression of other downstream genes (including *patS*) increase sequentially. As discussed earlier, PatS is a diffusible entity which continuously leaks out of that *patS* expressing cell to neighboring cells via the periplasmic space between the inner and outer membrane of the filament. *hetR* dominates over *patS* and continues the differentiation program in the developing cell. Neighboring cells within the distance that PatS peptide reaches, development is suspended thus establishing the pattern of heterocysts. The balance between the two gene products in two different cell types may switch the fate of the cell to be a heterocyst or a vegetative cell.

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