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Epistasis Analysis of Four Genes from *Anabaena* sp. Strain PCC 7120 Suggests a Connection between PatA and PatS in Heterocyst Pattern Formation

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The *hetR*, *patA*, *hetN*, and *patS* genes are part of a regulatory network that regulates the differentiation and patterning of heterocysts in the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120. In this report, the epistatic interactions of mutant alleles of these four genes have been used to refine our understanding of their relationships to one another. The *hetR* gene was necessary for differentiation in genetic backgrounds that normally give rise to excessive differentiation, supporting its role as the master regulator of differentiation and indicating that HetR directly regulates factors in addition to *hetR* and *patS* genes that regulate differentiation. A functional *patS* gene was necessary for the delayed multiple-contiguous-heterocyst phenotype observed in *hetN* mutants as well as for the relative lack of intercalary heterocysts in *patA* mutants. Epistasis results with mutant alleles of these three genes suggested that PatA attenuates the negative effects of both PatS and HetN on differentiation and promotes differentiation independent of its antagonistic effects on PatS and HetN activity. Cooverexpression of *patS* and *hetR* in a synthetic operon indicated that *patS* acts at a point downstream of *hetR* transcription in the regulatory network controlling differentiation. A model for the regulation of differentiation that is consistent with these and previous findings is presented.

Anabaena sp. strain PCC 7120 is a filamentous cyanobacterium that uses two distinct cell types to physically separate incompatible metabolic processes. Filaments are capable of both photosynthesis and dinitrogen fixation under aerobic conditions. Nitrogenase, the nitrogen-fixing enzyme complex, is O_2 labile and must be sequestered away from processes that evolve O_2. In the presence of a source of combined nitrogen, such as nitrate or ammonia, unbranched filaments of 100 cells or more grow as undifferentiated chains of vegetative cells. In contrast, when *Anabaena* sp. strain PCC 7120 is deprived of combined nitrogen, approximately every 10th cell of the filament develops into a morphologically distinct heterocyst that supplies the rest of the filament with fixed nitrogen and in return receives carbohydrate from vegetative cells (33). The heterocysts are evenly spaced along the filament to form a semiregular pattern. De novo pattern formation and commitment of the cell's fate are determined between 9 and 14 h after induction under laboratory conditions, and functional heterocysts have formed after 18 to 24 h (36). As the filament grows and the number of vegetative cells between heterocysts increases, single vegetative cells midway between two fixing cells develop into heterocysts, thus maintaining the pattern of differentiated cells.

Several genes that are involved in differentiation and patterning have been identified (for a review, see references 18 and 31). Four of these, *hetR*, *patA*, *patS*, and *hetN*, appear to encode central regulatory factors. The *hetR* gene is a positive regulator of differentiation; mutations that change either of two serine residues, both of which are necessary for protease activity of the peptide, result in a Het^- phenotype (6, 11, 38), and overexpression or extra copies of *hetR* in filaments result in a multiple-contiguous-heterocyst (Mch) phenotype, which is characterized by clusters of heterocysts separated by a reduced number of vegetative cells, even under conditions that do not induce heterocyst formation in the wild-type strain (6, 7). HetR has recently been shown to bind to DNA from the *hetR* and *patS* promoter regions. A cysteine-to-alanine conversion at amino acid 48 prevents DNA binding and dimerization of HetR in vitro, and cells containing only the corresponding mutant allele do not differentiate heterocysts (21). The regulation of *hetR* appears to be complex and is only beginning to be understood. There are multiple transcriptional start sites in the promoter of *hetR*, some of which require *hetR* and/or the transcriptional activator *ntcA*, which requires *hetR* for its own activation (7, 27). HetR from *Anabaena* sp. strain PCC 7120 displays a number of different isoelectric points, suggesting that HetR is modified posttranslationally, which may serve to regulate the activity of the protein (37). The requirement of *hetR* for differentiation, its ability to promote heterocyst formation in the absence of the differentiation signal, and its positive autoregulation and multiple points of regulation make it a candidate for the master regulator of differentiation.

In contrast to *hetR*, *patS* and *hetN* suppress heterocyst differentiation when present on a multicopy plasmid or when overexpressed from an inducible promoter. The gene *patS* governs de novo pattern formation when filaments are induced to differentiate. A *patS*-null mutant exhibits a Mch phenotype on nitrogen-deficient media and abnormal differentiation in the presence of nitrate (35). The gene encodes a 13- or 17-amino-acid peptide, and the exogenous addition of its C-terminal

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pentapeptide to a culture of *Anabaena* sp. strain PCC 7120 prevents the induction of heterocysts. It has been proposed that the PatS peptide diffuses away from differentiating proheterocysts along the filament to create a gradient of inhibitory signal that governs pattern formation (35). The receptor for the PatS signal is cytoplasmic (34), and the PatS C-terminal pentapeptide prevents the binding of HetR to regions of the *hetR* promoter (21), suggesting a direct action of PatS in the prevention of *hetR* autoregulation. A *hetR*(*R223W*) mutant allele has been shown to be insensitive to suppression by PatS and HetN (23), a second inhibitor of differentiation that affects patterning. Expression of *patS* is induced soon after nitrogen deprivation, but after differentiation is complete, expression of *patS* returns to preinduction levels (35). The fact that a *patS* deletion strain shifts from a Mch pattern of heterocysts to a more wild-type pattern after 72 h (36) suggests that another factor(s) is involved in pattern maintenance.

A second gene whose product suppresses heterocyst differentiation when in excess, *hetN*, encodes a putative ketoacyl reductase and is believed to play a role in the maintenance of heterocyst patterning (3). Unlike with a *patS* mutant, filaments that do not express *hetN* first develop a normal pattern of heterocysts at 24 h after induction. It is not until 48 h that excessive differentiation of heterocysts results in an Mch phenotype (10). The delay in the appearance of the Mch phenotype when *hetN* is turned off and the fact that *hetN* is normally not expressed until 12 h after induction (2) suggest that HetN does not play a major role in de novo heterocyst pattern formation. Instead, it appears to be necessary for maintenance of the pattern as filaments lengthen by cell growth and division and new heterocysts form between existing ones. A low level of HetN protein is present in vegetative cells under noninducing conditions, but after induction of differentiation, HetN protein is located exclusively in mature heterocysts (25) and expression of *hetN* is primarily in heterocysts (10). The putative HetN-dependent signal blocks heterocyst formation at points both upstream and downstream of *hetR* transcription; overexpression of *hetN* both prevents the patterned expression of *hetR* and prevents the Mch phenotype that normally results from ectopic expression of *hetR* from an inducible promoter. These findings have led to the suggestion that HetN inhibits heterocyst formation by blocking *hetR*-positive autoregulation (10). Simultaneous inactivation of *patS* and *hetN* leads to the complete differentiation of all cells of a filament into heterocysts (5).

The gene *patA* was discovered in 1992, and both its mutant phenotype and its role in heterocyst formation have remained an enigma for the past 13 years. It encodes a putative two-domain protein whose C-terminal region is similar in sequence to CheY and the N-terminal receiver domains of response regulators involved in two-component signal transduction systems (26). The conservation of three amino acids necessary for phosphorylation of CheY, including the aspartate modified to regulate CheY activity (28), suggests that regulation of PatA may involve phosphorylation. The N-terminal half of PatA is similar only to a few other PatA-like proteins from cyanobacteria. Mutants rarely form intercalary heterocysts but do differentiate single heterocysts at the terminal positions of filaments regardless of the length of the filament. Intercalary heterocysts, although rare, are sometimes observed in *patA* mutants, suggesting that a unique differentiation process in terminal cells that does not require *patA* is not responsible for the phenotype. The effect of overexpression of *hetR* from an inducible promoter, which gives an Mch phenotype in the wild type, is suppressed in these mutants (a *patA* mutant overexpressing *hetR* has a *patA* phenotype), suggesting that PatA acts downstream of the transcription of *hetR* in the regulatory network controlling differentiation (7, 26). Induction of the transcription of *hetR* in only the terminal cells of a *patA* mutant indicates that PatA also influences differentiation upstream of the transcription of *hetR* (7). There is a modest increase in *patA* mRNA levels between 3 and 6 h postinduction, but the *patA* gene transcript is present at very low levels in media with or without fixed nitrogen (26).

Genetic epistasis analysis can be used to gain insight into functional relationships between genes and their roles in regulatory pathways without knowledge of all the genes in a pathway or the molecular details of the gene product functions (1). In this report, the phenotypes of strains with single mutations in the *hetR*, *patA*, *hetN*, and *patS* genes are compared to those of strains carrying mutations in two or three of these genes. The epistatic interactions of the mutant alleles of the *hetR*, *patA*, *hetN*, and *patS* genes have been used to refine our understanding of their relationships to one another with regard to heterocyst differentiation and patterning, and a hypothesis is offered for the role of *patA* based on the mutant phenotypes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Growth of *Escherichia coli* and *Anabaena* sp. strain PCC 7120 strains, concentrations of antibiotics, induction of heterocyst formation using BG-11_0 medium, which lacks a combined nitrogen source, and conditions for photomicroscopy were as previously described (5). To determine average lengths of vegetative-cell intervals, the number of vegetative cells between two noncontiguous heterocysts was determined for 50 intervals at the time points indicated in Fig. 3. Except with the *patA* mutant UHM101 (which was fragmented with a Branson 1510 ultrasonic cleaner to a length equal to that of the average vegetative-cell interval of the *patA patS* double mutant UHM113), at least one heterocyst was required to be intercalary in order to account for any bias that might result from short filaments containing two terminal heterocysts. To determine the percentage of multiple-contiguous-heterocyst formations, 100 heterocysts (or heterocyst clusters) were counted and the number of contiguous heterocysts was recorded for each. All results are represented as averages from three replicates, with error bars indicating 1 standard deviation.

Plasmid constructions. All plasmids and oligonucleotides used in this study are described in Table 1. Plasmid pSMC136 was used to delete the *patA* gene from strains PCC 7120, UHM100 (5), and 7120PN (10) to create strains UHM101, UHM108, and UHM112, respectively. A 833-bp region of DNA located 820 bp upstream of the *patA* coding region and a 918-bp region of DNA starting 13 bp upstream of the *patA* stop codon were amplified from the chromosome (using primers patAproF, patA5R2, patA3F2, and patA3R) and fused by overlap extension PCR as described previously (10, 20). The resulting fragment was cloned into the TA site of plasmid pGEM-T Easy (Promega), moved to pBluescript SK+ (Stratagene) as a NotI fragment, and subsequently moved to pRL278 (8) as a BamHI-SacI fragment to create pSMC136.

Plasmid pSMC164 was used to delete the *patS* gene from strains UHM101 and UHM103 to form strains UHM113 and UHM111, respectively. pSMC164 is a derivative of pSMC147 (5), which contains an unmarked deletion of *patS*. To create pSMC164, a SmaI fragment containing an Ω Sp^r/Sm^r interposon (13) was moved into the EcoRV site of pSMC147.

Plasmid pDR120 is a mobilizable shuttle vector that was used to overexpress *hetR* from the copper-inducible *petE* promoter. The 342-bp *petE* promoter and a 920-bp fragment corresponding to the *hetR* coding region with six histidine codons added to the 3′ end were amplified via PCR (using primer pairs PpetEF and petE-NdeI-down and hetRcf-NdeI and hetR6H-r, respectively) and cloned separately into the TA cloning vector pGEM-T (Promega). P_{petE} was moved into pBluescript as an ApaI-PstI fragment, and *hetR* was subsequently moved into this vector as an NdeI-PstI fragment using NdeI sites created by the primers at the 3′

TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristic(s)	Source or reference
Anabaena strains[a]		
PCC 7120	Wild type	Pasteur Culture Collection
UHM100	P_{petE}-*hetN* Δ*patS*	5
UHM101	Δ*patA*	This study
UHM103	Δ*hetR*	5
UHM108	P_{petE}-*hetN* Δ*patS* Δ*patA*	This study
UHM109	Δ*hetR* Δ*patA*	This study
UHM110	P_{petE}-*hetN* Δ*hetR*	This study
UHM111	Δ*hetR* Δ*patS*::Ω Sp^r/Sm^r	This study
UHM112	P_{petE}-*hetN*, Δ*patA*	This study
UHM113	Δ*patA* Δ*patS*::Ω Sp^r/Sm^r	This study
UHM114	Δ*patS*	5
UHM115	Δ*hetN*::Ω Sp^r/Sm^r	5
7120PN	P_{petE}-*hetN*	10
Plasmids		
pSMC104	Suicide plasmid used to delete *hetR*	5
pSMC136	Suicide plasmid used to delete *patA*	This study
pSMC147	Suicide plasmid used to delete *patS*	5
pSMC164	Suicide plasmid used to replace *patS* with an Ω Sp^r/Sm^r interposon insertion	This study
pDR120	Shuttle vector carrying P_{petE}-*hetR*	This study
pDR151	Shuttle vector carrying P_{petE}-*hetR patS*	This study
Oligonucleotides[b]		
patAproF	GGA CAA GTG AAT CAA TTC AAT GCT AA	
patA5R2	GTA ATA GTT GAT AGG CGA TCG CTC TGT TAT TAA ATC	
patA3F2	AGA GCG ATC GCC TAT CAA CTA TTA CCA ATT ACC CAT TAC C	
patA3R	TTT CTG CAG GGC TGA ACC TTT GGG AGT TAG TGG AG	
PpetEF	GCTGAGGTACTGAGTACACAGCTAAT	
petE-NdeI-down	CAT ATG GTT CTC CTA ACC TGT AGT TTT ATT TTT C	
hetRcf-NdeI	CAT ATG AGT AAC GAC ATC GAT CTG ATC	
hetR6H-R	TTA GTG ATG GTG ATG GTG ATG ATC TTC TTT TCT ACC AAA CAC CAT TTG	
patSF-BamHI	GGA TCC GTT CTG TTG AAA AGT AAT TCA CCG	
patSR-SacI	GAG CTC CCA AGT GGA TGA CTT TAT TTC CG	

[a] For *Anabaena* strains constructed in this study, mutations were introduced in the order indicated.
[b] Oligonucleotides read in the 5′-to-3′ direction.

end of P_{petE} and the 5′ end of *hetR* to create pDR119. The P_{petE}-*hetR* fusion was moved from pDR119 to pAM504 (30) as a KpnI-SacI fragment to create pDR120.

Plasmid pDR151 differs from pDR120 by the addition of a 308-bp fragment starting 35 bp upstream of the *patS* coding region generated by PCR (using primers patSF-BamHI and patSR-SacI). The 308-bp fragment was cloned into pGEM-T and moved to pDR119 containing the P_{petE}-*hetR* fusion as a BamHI-SacI fragment using restriction sites incorporated into the primers. The resulting P_{petE}-*hetR patS* synthetic operon was subsequently cloned into pAM504 as a KpnI-SacI fragment to create pDR151.

All PCR products were verified by sequencing in pGEM-T derivatives prior to subsequent manipulation.

Strain constructions. All *Anabaena* sp. strain PCC 7120 strains used in this study are described in Table 1. Clean, unmarked deletions were introduced to make all of the double-deletion mutants except UHM111 and UHM113, which, in place of the deleted region, contain an Ω interposon that confers resistance to spectinomycin and streptomycin. Mutant strains were created essentially as previously described (8, 10), with the following exceptions. Plasmids were conjugated into the appropriate strain, and cells that incorporated the plasmid into the chromosome were selected on neomycin, except for plasmid pSMC164, which was selected on spectinomycin and streptomycin. Single recombinants were grown in nonselective liquid culture and subcultured twice. To avoid a mixed culture within a filament, filaments were fragmented to mostly single cells using a Branson 1510 ultrasonic cleaner once prior to liquid subculturing and again prior to plating on solid BG-11 medium containing 5% sucrose to select for the loss of the vector-borne *sacB* gene. The loss of the vector was verified by sensitivity to neomycin, resistance to which is encoded by the vector. UHM111 and UHM113 were selected on BG-11 medium containing 5% sucrose, spectinomycin, and streptomycin. To confirm mutant constructions, primers flanking the mutation and located outside the region of *Anabaena* sp. strain PCC 7120 DNA used on the suicide plasmid to make the mutation were used to amplify the region of the intended mutation. The sizes of the PCR products were used to confirm that the mutant construct had replaced the wild-type region of DNA. For initial screening of unmarked mutations in the double recombinants, colony PCR was used. In place of purified DNA, 2 μl of a suspension of filaments in water with an absorbance of approximately 0.3 at 750 nm and incubated at 100°C for 2 min was used as the source of template DNA. Chromosomal alterations were made in the order indicated in Table 1.

RESULTS AND DISCUSSION

Mutant strains. To facilitate the examination of the phenotypic effects of genetic background on mutations in genes necessary for proper patterning of heterocysts, several derivatives of *Anabaena* sp. strain PCC 7120 were created with defined single, double, or triple mutations in the *hetR*, *patA*, *hetN*, and *patS* genes (Table 1). For this study, mutations that prevent gene expression were used to ensure a null phenotype in the single mutants and to rule out unpredicted interactions in the double mutants used in the epistasis analysis. Mutations known to prevent the formation of a protein product are necessary to prevent the potential effects of a seemingly nonfunctional protein. For instance, in *Anabaena* sp. strain PCC 7120, a mutant

allele of *hetR* that has a point mutation causing a serine-to-asparagine conversion at residue 179 was shown 13 years after its isolation to be dominant to the wild-type allele when both are present within a cell (21). In other systems, truncated peptides that can be generated by insertion, nonsense, and frameshift mutations have been shown to have altered activities (e.g., reference 12), and transposon insertions have been shown to affect the regulation of nearby genes (29). Each of the mutations used in this study is either a clean deletion of the gene, a replacement of the gene with an interposon that confers resistance to both spectinomycin and streptomycin, or a replacement of the promoter region of a gene by the copper-inducible *petE* promoter, which can be conditionally down-regulated to prevent expression.

For mutations of *hetR*, *patA*, and, in some strains, *patS*, clean deletions of the coding regions were made via gene replacement without the addition of exogenous DNA. When used for the inactivation of monocistronically encoded genes, this type of mutation has the least potential to affect the expression of surrounding genes and does not preclude the use of any antibiotic-resistance markers in subsequent manipulations. Although the creation of such mutations is common in some bacteria and has been done in another filamentous cyanobacterium, *Nostoc punctiforme* ATCC 29133 (19), it is exceptional in *Anabaena* sp. strain PCC 7120. The unmarked mutations in this study were made using a previously described procedure (8) modified slightly to accommodate the lack of selection for the desired mutations. In particular, single recombinants were grown for a prolonged period of time to allow complete segregation of the unmarked mutations within single cells, and filaments were fragmented twice prior to selection for the second recombination event to avoid mixed-population filaments. Colony PCR was then used to screen for filaments in which the second recombination event had yielded the desired genotype. With these minor modifications, approximately half of the double recombinants tested were found to have the desired mutations. The modified procedure allows the clean deletion or replacement (10) of DNA and the creation of single-base-pair substitution mutations (D. Risser and S. M. Callahan, unpublished) in the chromosome of *Anabaena* sp. strain PCC 7120. It should also facilitate the creation of nonpolar deletion mutations in operons.

In *hetR*-null mutants, the coding and promoter regions were removed from the chromosome. Unlike the wild type, which forms a semiregular pattern of heterocysts in BG-11_0 (Fig. 1A), the *hetR* deletion single mutant strain UHM103 is Het^- under all conditions of growth, consistent with the phenotype of previously described substitution and insertion mutants (Fig. 1B) (4, 6, 11). In *patA*-null mutants, the coding region has been deleted cleanly from the chromosome. The *patA* deletion single mutant strain UHM101 forms predominantly terminal heterocysts and is indistinguishable from a previously described replacement mutant (Fig. 1C) (26). In the *patS*-null mutants described herein, a previously described region of 381 bp containing the *patS* gene (35) was either deleted cleanly from the chromosome or replaced by an Ω Sp^r/Sm^r cassette, depending on the strain. The *patS* deletion single mutant strain UHM114, which does not contain the Ω Sp^r/Sm^r cassette, is Mch in BG-11_0 and appears indistinguishable from a previously described *patS* replacement mutant (Fig. 1D) (35, 36). To inactivate *hetN* in each of the strains, we used a conditional allele of *hetN* that has the normal chromosomal promoter replaced by the copper-inducible *petE* promoter (10). When copper is omitted from the medium, transcription from the *petE* promoter ceases (17). Omission of copper does not affect heterocyst differentiation or patterning in *Anabaena* sp. strain PCC 7120, the wild-type strain (10). The P_{petE}-*hetN* single mutant 7120PN is Mch in BG-11_0 lacking copper, but unlike with the *patS* mutants, the degree of Mch is not as extreme, and the appearance of the Mch phenotype is delayed approximately 24 h (Fig. 1E) (10). The phenotype of 7120PN in the absence of copper and a source of fixed nitrogen is indistinguishable from that of strain UHM115, which has the *hetN* gene replaced by an Ω Sp^r/Sm^r cassette (5).

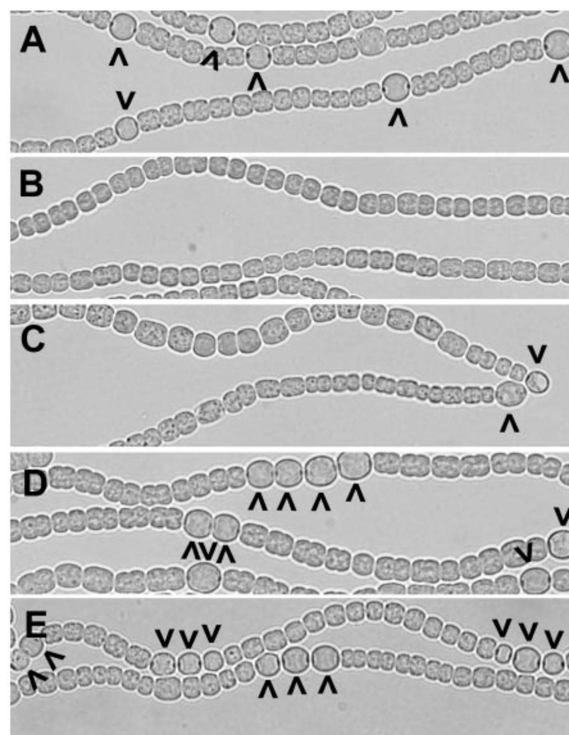

FIG. 1. Phenotypes of single mutant strains 48 h postinduction. PCC 7120 (A), *hetR* mutant strain UHM103 (B), *patA* mutant strain UHM101 (C), *patS* mutant strain UHM114 (D), *hetN* mutant strain 7120PN (E).

In addition to the single mutants, double mutants with all six possible pairwise combinations of the *hetR*, *patA*, *hetN*, and *patS* mutations were constructed to compare the single and double mutant phenotypes and to determine possible epistatic interactions. The phenotypes of five double mutants and one triple mutant are described in this report. The phenotype of a sixth double mutant, a P_{petE}-*hetN* Δ*patS* mutant, has been reported previously (5). Because each of the mutations results in a distinct phenotype with respect to heterocyst formation and patterning, it is possible in some cases to establish which mutation is epistatic based on the phenotype of the double mutant.

Deletion of the *hetR* gene is epistatic to *patS*, *patA*, and *hetN* mutations. There is extensive evidence that *hetR* is the master regulator of differentiation in *Anabaena* sp. strain PCC 7120

and is required for positive regulation of heterocyst formation. Because no positive regulatory factors other than *hetR* itself have been shown to be directly regulated by HetR, it is possible that one major function of HetR is the inhibition of a suppressor of differentiation, such as PatS or HetN, one or both of which regulate the differentiation process directly. If this scenario were true, inactivation of *patS* or *hetN* would be epistatic to inactivation of *hetR*. On the other hand, if HetR directly regulates other factors necessary for differentiation and is absolutely required for differentiation, inactivation of *hetR* should be epistatic and the double mutant phenotypes should be Het$^-$. Strains UHM109, UHM110, and UHM111 have mutations in the *patA*, *hetN*, and *patS* genes, respectively, in a Δ*hetR* background. Each of the double mutant strains had a Het$^-$ phenotype indistinguishable from that of strain UHM103, the single-*hetR*-deletion strain, indicating that deletion of *hetR* is epistatic to the other three mutations (data not shown), thus ruling out the possibility that the primary role of HetR in heterocyst differentiation is to prevent suppression of differentiation by HetN or PatS. Given the role proposed for PatA below, it is not surprising that the terminal heterocysts produced by a *patA* mutant require a functional *hetR* gene.

Epistasis of the *hetR* deletion reinforces the concept of HetR as an indispensable positive regulator of the heterocyst formation that is required even in genetic backgrounds that otherwise cause an excessive or altered pattern of differentiation. HetR is required for induction of the expression of six regulatory genes in response to diazotrophic conditions, *ntcA* (27), *patS* (21), *hetR* (4), *hetP* (14), *hetC* (22), and *devA* (9), but direct interactions of HetR with the promoter regions of only *patS* and *hetR* have been demonstrated (21). The epistasis results and our failure to isolate mutations that suppress the Het$^-$ phenotype of the *hetR* deletion mutant strain UHM103 and similar attempts by others (6; S. M. Callahan, unpublished data) indicate that *hetR* directly regulates the activity of factors in addition to *hetR* and *patS* genes that are necessary for differentiation.

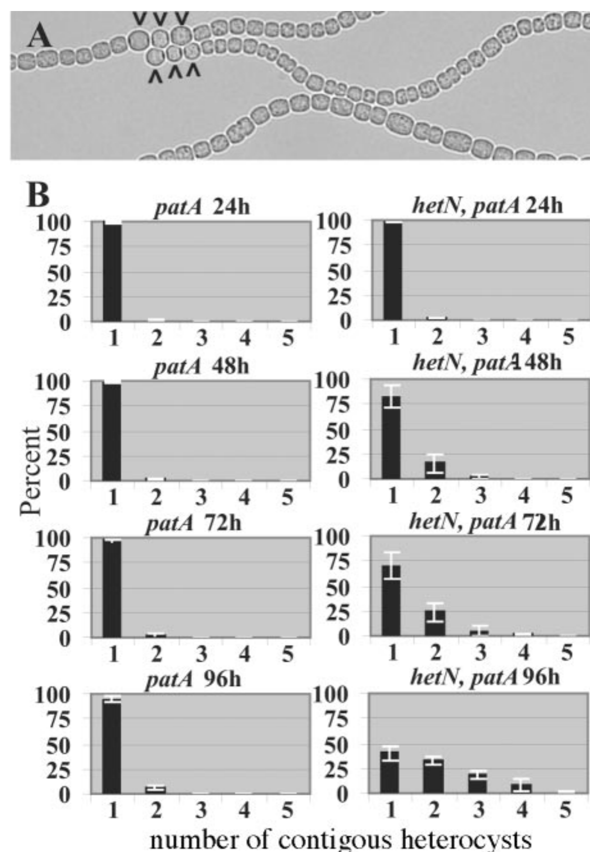

FIG. 2. Mch termini of a *hetN patA* double mutant strain. UHM112 48 h postinduction in copper-free medium (A). Comparison of the percentages of filament termini from strains UHM101 and UHM112, which have the indicated numbers of contiguous heterocysts at different times postinduction (B).

In a Δ*patA* background, inactivation of *hetN* leads to multiple contiguous heterocysts at the filament termini. Inactivation of *hetN* in a wild-type genetic background causes a delayed Mch phenotype when filaments are induced for heterocyst formation; 24 h after the removal of fixed nitrogen, the pattern appears wild type, but at 48 h, it is Mch (Fig. 1E) (10). Strain UHM112 carries a deletion of the *patA* gene, and the copper-inducible allele of *hetN*, which can be inactivated by growth in media lacking copper, has replaced the wild-type copy in the chromosome (Table 1). With *hetN* inactivated in a Δ*patA* background, strain UHM112 had a delayed Mch phenotype primarily at the filament termini when the strain was induced for heterocyst formation in medium lacking fixed nitrogen. At 24 h postinduction, the phenotype was indistinguishable from that of a *patA* mutant, but at approximately 48 h postinduction and thereafter, many of the filament termini had multiple heterocysts (Fig. 2A). The percentage of filament termini that were Mch and the extent of the Mch phenotype increased with time (Fig. 2B). In addition, the rare intercalary heterocysts that did form were often part of a cluster of heterocysts at 48 h and thereafter.

A delay of 24 h in the appearance of the Mch phenotype is unique to strains that have a mutation in *hetN* and is approximately equal to the time necessary for differentiation of a vegetative cell into a heterocyst. The delay in the formation of additional heterocysts adjacent to existing terminal heterocysts in strain UHM112 could, therefore, be dependent on (i) the production of a positive signal for differentiation from mature heterocysts or (ii) the cessation of production of a suppressor of differentiation when a heterocyst matures. Yoon and Golden used a *patS-lacZ* translational fusion and Northern blot analyses to show that production of PatS decreases to preinduction levels at approximately the same time that mature heterocysts appear (35). PatS and HetN have been shown to act independently (5), so PatS is functional in *hetN* mutants and is a likely candidate for the suppressor of the differentiation responsible for the delay in the appearance of the Mch phenotype of *hetN* mutants, as described in the second scenario above. If correct, this hypothesis predicts that deletion of the *patS* gene from strain UHM112 would eliminate the delay in the appearance of the terminal Mch phenotype.

Formation of intercalary heterocysts in a *patA* genetic background by a *patS hetN patA* triple mutant. To determine whether the delay in the appearance of the terminal Mch phenotype of strain UHM112 is due to the production of PatS in filaments, strain UHM108, which has both *patA* and *patS* deleted from the chromosome and *hetN* under the control of a conditional promoter, was constructed. At 24 h postinduction,

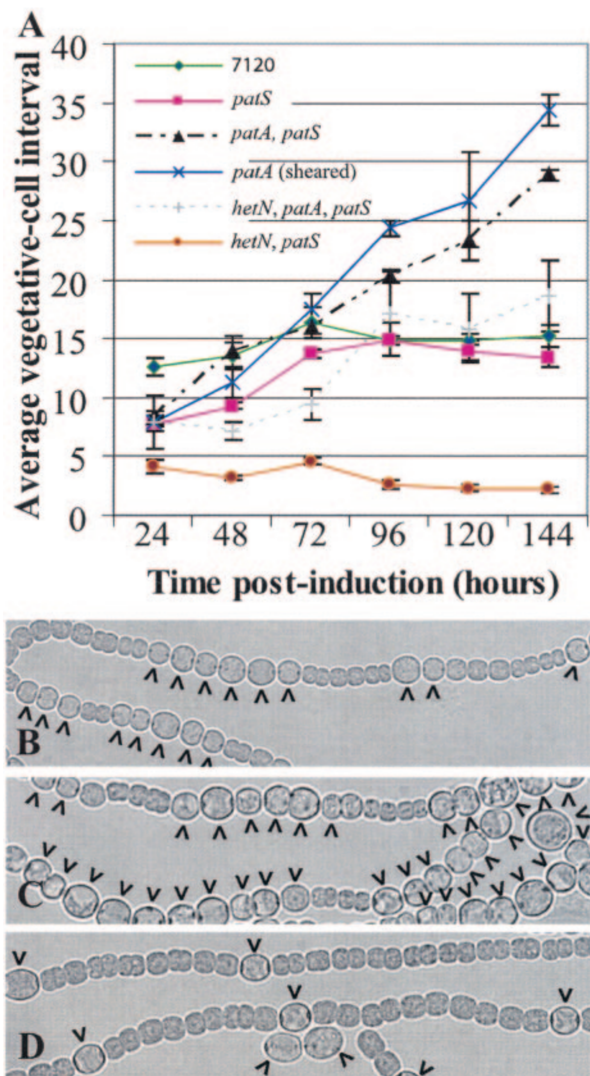

FIG. 3. Vegetative-cell intervals versus time postinduction and the phenotype of a *patS hetN patA* mutant. Average numbers of vegetative cells between clusters of heterocysts (average vegetative-cell interval) at various times postinduction (A). PCC 7120 (◆), UHM114 (■), UHM113 (▲), UHM101 sheared to an average filament length of 10 cells prior to induction (✕), UHM108 (✚), UHM100 (●). UHM108 48 h postinduction in copper-free medium (B). UHM100 48 h postinduction in copper-free medium (C). UHM108 in copper-free medium containing nitrate (D).

strain UHM108 was Mch at both the intercalary and terminal positions of its filaments, and the average number of vegetative cells between clusters of heterocysts (average vegetative-cell interval) was reduced compared to that of the wild type (Fig. 3A and B). The number of heterocysts and the reduction in the length of vegetative-cell intervals is not as extreme as in the *patS hetN* double mutant UHM100 (Fig. 3C) (5). In the triple mutant, reduced vegetative-cell intervals persisted until 72 h postinduction. At 96 and 120 h postinduction, vegetative-cell intervals resembled those in the wild type, and at 144 h and thereafter, vegetative-cell intervals exceeded those of the wild type and continued to increase slowly with time to approximately 30 at 240 h postinduction, the last time point recorded.

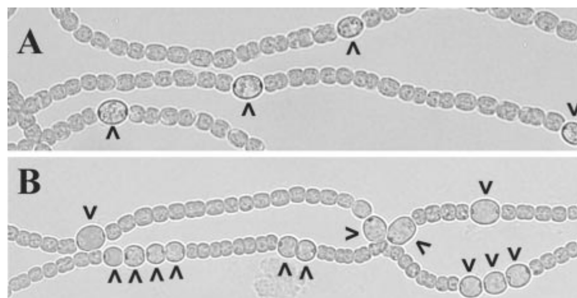

FIG. 4. Heterocyst formation by a *patS patA* double mutant. UHM113 in medium containing nitrate (A). UHM113 24 h postinduction (B).

In addition to forming intercalary heterocysts in media lacking fixed nitrogen, UHM108 formed single heterocysts at both the intercalary and terminal positions of its filaments in BG-11, which contains nitrate (Fig. 3D).

The lack of a delay in the appearance of the Mch phenotype in the triple mutant strain UHM108 demonstrates that the delay of approximately 24 h in the appearance of an Mch phenotype in the *patA hetN* mutant strain UHM112 is dependent on a functional *patS* gene. The correlation between the timing of the appearance of additional heterocysts and the downregulation of *patS* upon heterocyst maturation suggests that expression of *patS* in proheterocysts is responsible for the delayed Mch phenotype of *hetN* strains in general. The lack of a delay in the appearance of an Mch phenotype in strain UHM100, which is a P_{petE}-*hetN* Δ*patS* double mutant (5), is consistent with this idea.

All strains carrying a mutation in the *patA* gene described to date display an enigmatic phenotype characterized by the formation of primarily terminal heterocysts when induced to differentiate. However, the triple mutant described here, strain UHM108, formed intercalary heterocysts in a *patA* genetic background. Because the P_{petE}-*hetN patA* double mutant UHM112 retained the terminal-heterocyst phenotype, the difference in genetic complements between strains UHM108 and UHM112 suggested that the phenotype was dependent primarily on inactivation of *patS*.

A *patS patA* double mutant resembles a *patS* single mutant 24 h postinduction. To determine whether inactivation of *patS* in a *patA* background restores the ability of a *patA* mutant to form intercalary heterocysts, strain UHM113 was created by replacing the *patS* gene of strain UHM101 with an Ω Sp^r/Sm^r cassette to create a *patS patA* double mutant. UHM113 resembled a *patS* single mutant in media containing nitrate. Single heterocysts located at both the terminal and intercalary positions were separated by stretches of vegetative cells, the same phenotype observed for strain UHM114, and a previously described *patS* replacement mutant (Fig. 4A) (35). At 24 h postinduction, strain UHM113 formed both intercalary and terminal heterocysts. The Mch phenotype of this strain at 24 h was indistinguishable from that of a *patS* single mutant for both the number of heterocysts formed and the length of vegetative-cell intervals between clusters of heterocysts (Fig. 3A and 4B).

These results indicate that a functional *patS* gene is necessary for the formation of the *patA* phenotype. A hypothesis that accounts for the unusual *patA* phenotype and the apparent

epistasis of *patS* at 24 h postinduction predicts a role for *patA* in attenuation of the *patS* signal responsible for suppression of differentiation. The model of lateral inhibition by a *patS*-dependent inhibitor of differentiation proposed to regulate initial pattern formation (35) implies that individual cells receive inhibitor from neighboring cells. In this model, intercalary cells receive a diffusible inhibitor of differentiation from neighboring cells on two sides while terminal cells receive inhibitor from only one direction. Thus, the functional concentration of inhibitor in intercalary cells would be twice that in terminal cells. Our results support a role for PatA in attenuation of the effect of the *patS*-dependent inhibitor that allows intercalary heterocysts to form. Without PatA attenuating the diffusible inhibitor, the concentration is high enough in intercalary cells to prevent heterocyst formation. However, in terminal cells, where the concentration of inhibitor is about half that in intercalary cells, PatA is not required for differentiation under diazotrophic conditions. We propose that one of the functions of PatA in heterocyst differentiation is to allow a cell to overcome suppression of differentiation by a PatS-dependent signal.

The hypothesis of the PatA function described above presumes that the terminal cells of a filament experience half the level of a *patS*-dependent signal experienced by intercalary cells. If true, this presumption has several implications with respect to heterocyst pattern formation. First, the terminal cells of a filament would be expected to have an increased chance of becoming a heterocyst. This does, indeed, seem to be the case. In the wild type, 82% (data not shown) of terminal cells were heterocysts, compared to approximately 10% for intercalary cells. As early as 1967, work by C. P. Wolk implied that terminal cells have an increased chance of differentiating (32). Second, preferential heterocyst formation at the terminus of a filament may serve as a frame of reference for pattern formation by nearby cells and, perhaps, for the entire filament. Third, a mutation that boosts the production of, or sensitivity to, a diffusible inhibitor by a factor of 2, for instance, would result in the formation of heterocysts only at the terminal cells, which would now receive an inhibitory signal at approximately the level of that received by intercalary vegetative cells in the wild type. The *patS patA* double mutant phenotype suggests that *patA* attenuates the effect of a *patS*-dependent signal during initial pattern formation.

Growth of vegetative-cell intervals by a *patS patA* double mutant resembles that of a *patA* single mutant after 24 h postinduction. The phenotypes of the *patS* single and *patS patA* double mutants were indistinguishable in media containing nitrate as the nitrogen source and at 24 h postinduction, but at 48 h postinduction and thereafter, they differed. Vegetative-cell intervals in a *patS* mutant are shorter than those in the wild type until about 72 h postinduction, when they become similar and remain so (Fig. 3A) (36). Conversely, lengths of vegetative-cell intervals in the *patS patA* double mutant increased to equal those in the wild type by 48 h postinduction and continued to lengthen thereafter (Fig. 3A). In fact, filaments of UHM113 that were under diazotrophic conditions for a prolonged period of time resembled a *patA* mutant under the same conditions, with the exception that many of the filament termini were Mch. Shearing of filaments during growth at heterocyst-heterocyst and heterocyst–vegetative-cell junctions, which appear to be more fragile than vegetative-cell–vegetative-cell junctions, combined with a lack of intercalary-heterocyst formation, probably accounted for the terminal-heterocyst phenotype. To test whether heterocyst formation between two existing heterocysts in strain UHM113 after 24 h postinduction is as rare as intercalary-cell differentiation in a *patA* single mutant, filaments of the *patA* mutant UHM101 were sheared to an average length approximately equal to the average vegetative-cell interval between heterocysts in strain UHM113 at 24 h postinduction, and the average vegetative-cell interval between the two heterocysts that formed at the ends was recorded over time. Vegetative-cell intervals for strain UHM113 and sheared UHM101 continuously increased over time for both strains in similar manners, although intervals in UHM101 were slightly longer than those in UHM113 after 72 h postinduction. The *patS patA* double mutant strain UHM113, therefore, had a *patS* phenotype at 24 h postinduction and appeared to resemble a *patA* mutant thereafter with respect to the comparative lack of differentiation of cells between two existing heterocysts.

The *patA*-like vegetative-cell intervals of the *patS patA* double mutant strain UHM113 at 48 h postinduction were not due to an excess of *patS*-dependent signal as proposed earlier for *patA* single mutants because *patS* is not active in the former strain. Instead, the phenotype is dependent on a functional *hetN* gene because when *hetN* was inactivated in a *patS patA* background, strain UHM108 had fewer vegetative-cell intervals at 48 h postinduction and thereafter than the *patS patA* double mutant strain. Therefore, deletion of *patA* appears to increase the ability of a HetN-dependent signal to prevent heterocyst differentiation. In other words, the *hetN*-dependent inhibition of differentiation caused by deletion of *patA* in a *patS* genetic background suggests that one of the functions of PatA in heterocyst differentiation is to allow a cell to overcome suppression of differentiation by a HetN-dependent signal. This interaction was, presumably, not noticed in the direct epistasis analysis of *hetN* and *patA* as it was for *patS* and *patA* because its effect was masked by the presence of PatS, which acts earlier in the differentiation process than does HetN. The epistasis results are supported by the recent finding that increased levels of expression of *patA* can overcome the suppression of differentiation by *hetN* (S. Y. Robbins and S. M. Callahan, unpublished data).

There are a number of possibilities as to the nature of the *patS*- and *hetN*-dependent signals and the attenuation of their effects by *patA*. PatA may directly interact with PatS- or HetN-dependent signals, resulting in their degradation, modification, or sequestration in source cells, in cells along the filament to create a concentration gradient, or in target cells. PatA may negatively regulate *patS* or *hetN* transcription in source cells. Finally, PatA may modify or interact with HetR in such a way as to make HetR less sensitive to inhibition by PatS or a HetN-dependent signal in proheterocysts. The recent isolation of an allele of *hetR* that yields a *patA*-like phenotype when used to complement the *hetR* deletion strain UHM103 or the *hetR patA* double deletion strain UHM109 adds weight to this last possibility (D. Risser and S. M. Callahan, unpublished data).

If the only function of *patA* is to decrease the sensitivity of HetR to PatS and HetN signals, the phenotypes of strains UHM108 and UHM100 should be identical, as should those of strains UHM113 and UHM114. However, the P_{petE}-*hetN*

Δ*patS* Δ*patA* triple mutant formed fewer heterocysts than the P_{petE}-*hetN* Δ*patS* double mutant, indicating that the function of PatA in promoting heterocyst differentiation extends beyond the attenuation of *patS*- and *hetN*-dependent signals. The allele of *hetR* capable of recreating a *patA*-like phenotype and an allele of *hetR* that is insensitive to *patS* or *hetN* overexpression and the exogenous addition of PatS-5 peptide (23) suggest that the function of PatA is mediated by HetR and that PatA also promotes differentiation independently from its effects on PatS and HetN activity.

Overexpression of *patS* is epistatic to overexpression of *hetR*. When *hetR* is overexpressed in the wild-type strain from the copper-inducible *petE* promoter on a plasmid, the resulting strain displays an Mch phenotype (7). On the other hand, overexpression of *patS* completely prevents heterocyst formation when it is overexpressed from the same promoter (35). In order to determine whether the inhibitory activity of *patS* on heterocyst formation acts downstream of *hetR* transcription, plasmids pDR120 and pDR151, which contain *hetR* or a synthetic operon comprised of both *hetR* and *patS*, respectively, under the control of the copper-inducible *petE* promoter, were introduced into both the wild-type strain and UHM111, a *patS hetR* double mutant. When strains containing either plasmid are cultured in the presence of copper, they overexpress either *hetR* alone or both *hetR* and *patS*. When cultured in media lacking copper, transcription from P_{petE} is turned off, and neither gene is expressed from the vectors. If the inhibitory activity of *patS* is limited to downregulating transcription of *hetR*, then bypassing the normal transcriptional regulation of *hetR* via the *petE* promoter should allow the formation of heterocysts even when *patS* is overexpressed. In media replete with copper, both strains containing pDR120 had an Mch phenotype as expected (data not shown). Conversely, both the wild-type strain and UHM111, a *patS hetR* double mutant containing pDR151, had no heterocysts. In both BG-11 and $BG\text{-}11_0$ media lacking copper, heterocyst formation in the wild-type strain containing pDR151 appeared similar to that of the same strain without pDR151 (data not shown). Strain UHM111 with or without pDR151 was unable to form heterocysts under either condition (data not shown). These results suggest that *patS* acts downstream of *hetR* transcription since bypassing native transcription of *hetR* via P_{petE} does not alleviate the negative effect of *patS* on heterocyst formation.

The fact that overexpression of *patS* is epistatic to overexpression of *hetR* shows that *patS* is capable of inhibiting heterocyst formation downstream of *hetR* transcription. Huang et al. have recently shown that HetR is a DNA-binding protein capable of binding to both the *hetR* and *patS* promoter regions when in a dimeric form and that the PatS pentapeptide is capable of blocking this DNA-binding activity (21). Along with the *hetR* epistasis results, the ability of excess PatS or HetN to prevent differentiation of a strain conditionally overexpressing *hetR* suggests that HetR directly regulates the activities of additional factors that are necessary for heterocyst differentiation.

Model of genetic interactions regulating heterocyst differentiation. Figure 5 is a model of the interactions between the genes investigated in this study and their effects on the regulation of differentiation. As depicted, the system has the potential to act as a biological switch. Biological switches convert

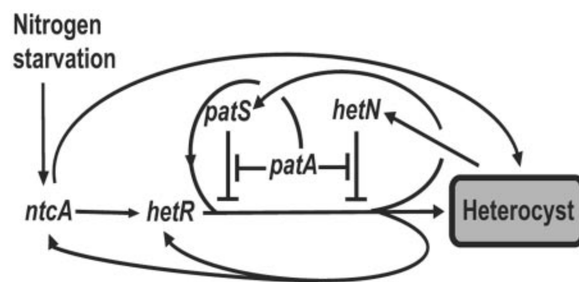

FIG. 5. Model depicting the genetic interactions of genes in this study and their roles in cell fate determination. See the text for details.

graded input signals into an all-or-none binary response and have been shown to regulate cell fate in developmental systems (16). The model shows the activation of *ntcA* by nitrogen starvation (probably as a result of increased cellular levels of 2-oxoglutarate, which enhance the DNA-binding activity of NtcA (24), which leads to the activation of a *hetR*-dependent differentiation pathway. The initial induction of *hetR* transcription via the indirect positive feedback loop is dependent on NtcA (27) and the nitrogen starvation signal, but subsequent induction is dependent exclusively on HetR via direct positive autoregulation (4), which would make the system both highly sensitive and self-perpetuating, the two other features of biological switches, even after the input signal is removed (15, 16). PatS acts nonautonomously to prevent the induction of the proposed biological switch to prevent excessive differentiation and govern pattern formation (35). As already mentioned, simultaneous inactivation of *patS* and *hetN* leads to complete differentiation of all cells of a filament into heterocysts (5). Both PatS and HetN are depicted preventing the activity of HetR at the same point, which is consistent with the recent description of an allele of *hetR* that codes for a protein insensitive to overexpression of both *patS* and *hetN* (23). Because overexpression of *patS* is epistatic to that of *hetR*, as demonstrated in this work, PatS, along with HetN, is depicted regulating the activity of *hetR* downstream as well as upstream of *hetR* transcription. The *hetR* epistasis results indicate that additional factors necessary for differentiation not pictured rely directly on HetR for their activity. PatA is shown attenuating the negative effects of both PatS and HetN on differentiation and promoting differentiation independent of its antagonistic effects on PatS and HetN activity, as suggested by the epistasis results. All three functions of PatA are probably mediated through HetR, although this is not depicted in the model.

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