

Different functions of HetR, a master regulator of heterocyst differentiation in *Anabaena* sp. PCC 7120, can be separated by mutation

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The HetR protein has long been recognized as a key player in the regulation of heterocyst development. HetR is known to possess autoproteolytic and DNA-binding activities. During a search for mutants of *Anabaena* sp. PCC 7120 that can overcome heterocyst suppression caused by overexpression of the *patS* gene, which encodes a negative regulator of differentiation, a bypass mutant strain, S2-45, was isolated that produced a defective pattern (Pat phenotype) of irregularly spaced single and multiple contiguous heterocysts (Mch phenotype) in combined nitrogen-free medium. Analysis of the S2-45 mutant revealed a R223W mutation in HetR, and reconstruction in the wild-type background showed that this mutation was responsible for the Mch phenotype and resistance not only to overexpressed *patS*, but also to overexpressed *hetN*, another negative regulator of differentiation. Ectopic overexpression of the *hetRR223W* allele in the *hetRR223W* background resulted in a conditionally lethal (complete differentiation) phenotype. Analysis of the heterocyst pattern in the *hetRR223W* mutant revealed that heterocysts differentiate essentially randomly along filaments, indicating that this mutation results in an active protein that is insensitive to the major signals governing heterocyst pattern formation. These data provide genetic evidence that, apart from being an essential activator of differentiation, HetR plays a central role in the signaling pathway that controls the heterocyst pattern.

patS | pattern formation | prokaryotic development | signal pathway

In *Anabaena* sp. strain PCC 7120, the first step in the establishment of aerobic diazotrophic growth is the formation of a pattern of cells that will differentiate into heterocysts. To understand how the heterocyst pattern is formed *de novo* upon nitrogen deprivation and maintained during continuous diazotrophic growth, it is crucial to identify several distinct groups of genes: those that positively regulate very early steps in development, the negative regulators of differentiation, and the components of signaling pathways that define when and where along the filament heterocysts should form.

Several genes have been shown to be involved in the early regulation of heterocyst development. The *ntcA* gene encodes a transcriptional regulator of the C-reactive protein family that controls a number of genes involved in nitrogen metabolism in cyanobacteria (1). Mutants lacking NtcA are pleiotropic and fail to show any signs of morphological differentiation upon nitrogen deprivation (2, 3). A mutant with a disrupted *hanA* gene encoding histone-like protein HU is also highly pleiotropic and is blocked in the earliest stage of heterocyst differentiation (4). Both *ntcA* and *hanA* are not heterocyst-specific genes, and there is no indication of their involvement in pattern formation.

The HetR protein has long been recognized as a major player in the regulation of heterocyst development (5, 6). Disruption of *hetR* by transposon insertion (6) and missense mutations S179N (5) and S152A (7) block the earliest stage of heterocyst differentiation without affecting growth on combined nitrogen. Overexpression of *hetR* in trans from its own promoters at a multicopy plasmid (5) or from the copper-inducible *petE* pro-

motor (8) leads to constitutive production of supernumerary heterocysts. HetR is an unusual serine-type protease, and two serine residues, Ser-152 and Ser-179, are required for both autoproteolysis and heterocyst differentiation, the Ser-152 apparently being the active site of the protease (7, 9). It has been recently found that HetR dimer binds specific DNA fragments containing promoter regions of *hetR*, *patS*, and *hepA* genes, and that its DNA-binding activity is inhibited by a synthetic PatS pentapeptide *in vitro* (10).

Two negative regulators, *patS* and *hetN*, in multicopy cause suppression of heterocyst differentiation and are required for normal heterocyst pattern formation and maintenance, respectively. Deletion of *patS*, which encodes a small peptide, results in an aberrant pattern and formation of multiple contiguous heterocysts (Pat Mch phenotype) upon nitrogen step-down (11). The phenotype becomes far less pronounced during continuous diazotrophic growth (12). The *hetN* product shows high sequence similarity to ketoacyl reductases (13, 14). Overexpression of *hetN* prevents up-regulation and patterned expression of *hetR* after nitrogen step-down (15, 16). HetN protein is present in vegetative cells grown with combined nitrogen, but after nitrogen step-down, it is found only in mature heterocysts (16). Depletion of HetN by switching off a copper-regulated promoter driving a chromosomal copy of *hetN* does not alter the initial heterocyst pattern, but causes a Mch phenotype during subsequent rounds of heterocyst formation (15). Thus, PatS and HetN have different but complementary functions in establishment and maintenance of the heterocyst pattern.

Several other genes may be related to the mechanism of pattern formation. In *Nostoc punctiforme*, the HetF protein is essential for triggering heterocyst differentiation (17). Similar to a *hetR* mutant, a *hetF* null mutant shows no sign of morphological differentiation or cell-specific degradation of phycobiliproteins 24 h after nitrogen step-down. Unlike *hetR*, extracopy *hetF* supplied on a replicative plasmid does not cause constitutive heterocyst production in ammonium- or nitrate-containing media. However, an increase in the frequency of heterocysts and production of short clusters of contiguous heterocysts were observed upon nitrogen deprivation, resembling the effect of extracopy *hetR* (5, 18). The HetF protein apparently typifies a unique family of caspase-hemoglobinase fold-domain proteases (19). Inactivation of *hetP*, whose product shows no homology to known proteins, resulted in a Het⁻ phenotype in *Anabaena* PCC 7120, whereas in multicopy, it caused production of Mch in combined nitrogen-free medium (20). PatA, a protein with a C-terminal domain similar to the CheY family of response regulators, is known to be essential for differentiation of intercalary, but not terminal, heterocysts (21). The *patA* mutation suppresses the Mch phenotype produced by extra copies of *hetR* (21) and confines *hetR* expression to terminal cells of a filament. It was postulated that PatA is required for the activation of HetR and

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increased expression of *hetR* in differentiating cells (8). PatB protein has ferredoxin-like domains near the N terminus and a DNA-binding domain near the C terminus, suggesting that PatB might be a redox-sensing transcription factor (22, 23). Its expression is induced 12 h after nitrogen step-down and is confined to heterocysts, and mutations in *patB* result in delayed heterocyst formation, poor or no diazotrophic growth (depending on the nature of mutation), and a gradually developing Mch phenotype, although initially formed heterocysts show a normal pattern (23).

Two more genes, *patN* and *patU*, were recently implicated in heterocyst pattern formation in *Nostoc punctiforme* (18). Both PatN and PatU have close orthologs in *Anabaena* PCC 7120, but show no similarity to other proteins. Mutations in *patU* result in strings of four to eight proheterocysts that ultimately resolve to single, double, or triple (Mch) heterocysts. A *patN* mutant yields a unique multiple singular heterocysts (Msh) phenotype, which is a high frequency of heterocysts located singly in the filaments.

The available genetic data indicate that aberrations in the heterocyst pattern can be obtained by altered expression or mutation of *hetR*, *hetP*, *hetN*, *hetF*, *patS*, *patA*, *patB*, *patU*, and *hetN* genes. In addition, high light intensity and certain chemicals (reviewed in ref. 18) cause production of Mch. However, no mutation producing random spacing of heterocysts has been reported. This led to a suggestion that the mechanism underlying the heterocyst pattern must either result from multiple redundant processes or be embedded in basic cellular functions and thus be immutable (18). Here, we present genetic evidence that a single mutation in *hetR* can produce a functional protein that is insensitive to the major signals governing heterocyst pattern formation.

Materials and Methods

Strains, Plasmids, and Growth Conditions. *Anabaena* sp. strain PCC 7120 and its derivatives were grown as described (24, 25). Mutant strains were grown in the presence of appropriate antibiotics at the following final concentrations: neomycin, 25 $\mu\text{g/ml}$ for solid medium and 15 $\mu\text{g/ml}$ for liquid medium; erythromycin, 5 $\mu\text{g/ml}$; spectinomycin, 5 $\mu\text{g/ml}$; streptomycin, 2.5 $\mu\text{g/ml}$. To induce heterocyst formation and diazotrophic growth, portions of early-growth-phase cultures were washed twice with BG-11₀ medium, which lacks combined nitrogen, and diluted 1:2 in BG-11₀. Scoring of the heterocyst pattern was performed as described (12). For heterocyst induction on agar plates, filaments from fresh streaks on BG-11 plates were patched on BG-11₀ plates and examined microscopically after 2–3 days of diazotrophic growth.

Microscopy. Bright-field and fluorescence microscopy were performed as described (12) except that an extra-long working-distance (ELWD) $\times 40$ objective (M Plan 40, 0.5 ELWD, 210/0, Nikon) was used, permitting visualization of vegetative cells and heterocysts in *Anabaena* filaments and of *gfp* expression directly on the surface of agar plates.

DNA Manipulations and Plasmid Constructions. Total DNA from cyanobacterial strains was extracted by vortexing cells with glass beads in the presence of phenol (26). Recombinant DNA procedures and enzyme reactions were performed by standard techniques or according to recommendations of the supplier. The mutant *hetR* allele was amplified from genomic DNA of strain AMC1285 by PCR with primers AMO-550 5'-GCAC-GACTAGTAAACAACACTGTTGC-3' (an engineered *SpeI* site is italicized) and AMO-551 5'-CGCCGAGTCATTTGTCATCACTAGC-3' using *Pwo* polymerase (Roche Applied Sciences). The amplified fragment was cut with *SpeI* and cloned into the *XbaI* site of pAM2178 (25) with *hetR* oriented parallel to *lacZ*, producing pAM3316, or into the *XbaI* site of pAM2179 (25) in the opposite orientation, producing pAM3317. These plasmids

contain the *hetR* upstream region starting at -181 , which is 2 nt 3' of the first -184 transcription start site mapped by Buikema and Haselkorn (8).

The *hetN* gene was overexpressed from the copper-inducible *petE* promoter on shuttle plasmid pAM1537, which was constructed as follows. The *hetN* gene was subcloned as a 1.3-kb *ClaI* fragment from cosmid clone 3E10 (13) into the *ClaI* site of pIC20H (27), such that *hetN* is in the opposite orientation relative to *lacZ*, to make pAM1531. The *petE* promoter was isolated from pPet1 (8) as a 372-bp *BamHI*–*EcoRI* fragment and then cloned into the same sites of pAM1531 to make pAM1533. A *BamHI*–*XhoI* fragment from pAM1533 containing P_{petE} –*hetN* was then cloned into the *BamHI*–*SalI* sites of shuttle vector pJL-3 (28) to make pAM1537.

Shuttle plasmid pAM1830 contains a *gfp* reporter downstream of *patS* as a transcriptional fusion and was constructed as follows. The *patS* ORF and ≈ 650 bp of upstream region was generated as a 0.72-kb *BamHI*–*SmaI* fragment by PCR of template pAM1035 (11) with primers 5'-CGCTCTAGAACTAGTG-GATC-3' (Bluescript SK) and 5'-CGCCGGGTCTATCTACCACTACCGC-3' followed by restriction enzyme digestion. This fragment was inserted into *XbaI* (filled in with Klenow polymerase) and *BamHI* sites of pKEN2-GFPmut2 (29) to make pAM1863. A *HindIII* (filled in with Klenow polymerase)–*SacI* fragment containing *patS*–*gfp* from pAM1863 was then cloned into *SacI*–*SmaI* sites of shuttle vector pAM505 (11) to make pAM1830.

For construction of a P_{petE} –*hetRR223W* transcriptional fusion, the Sp^r/Sm^r Ω cassette from pAM684 (30) was inserted into the *SmaI* site of the pAM3317 polylinker downstream of and parallel to *hetRR223W*, and the *HincII*–*SacI* fragment with *hetR*– Ω was moved into the *SmaI*–*SacI* sites of pPet1 (8) downstream of the P_{petE} promoter. Then the *PstI*–*SacI* fragment containing P_{petE} –*hetR*– Ω was moved into the same sites of shuttle vector pAM504 (3), producing pAM3318.

Results

Identification of a *hetR* Mutation Responsible for Aberrant Heterocyst Pattern. In an approach to clarify the functions of regulatory genes and unravel interactions among regulatory proteins during heterocyst differentiation and pattern formation, we isolated extragenic suppressors of the negative regulator PatS. During a search for transposon Tn5-1058-derived (31) mutants of *Anabaena* PCC 7120 that can overcome heterocyst suppression by extracopy *patS* supplied on plasmid pAM1888 (Em^r) (32), a bypass mutant strain, named S2-45, was isolated that produced long and irregularly spaced strings of multiple contiguous heterocysts in nitrogen-free medium (Fig. 1A). However, reconstruction of the transposon insertion in a wild-type background failed to reproduce the original phenotype, indicating the presence of a secondary mutation(s) in the original S2-45 bypass mutant.

To identify the mutation(s) responsible for the S2-45 (pAM1888) phenotype, the mutant strain was first cured of pAM1888 by repeated subculturing in liquid BG-11₀ medium without antibiotics and subsequent cloning. The cured strain, AMC1285, exhibited the original Pat Mch phenotype. To allow conjugative transfer of different pDU1-based plasmids carrying a Nm^r marker, the Nm^r Bm^r Sm^r genes on Tn5-1058 in the S2-45 chromosome were replaced with a Ω Sp^r Sm^r cassette. The resulting strain, AMC1286, exhibited the original Pat Mch phenotype.

We were interested in testing how AMC1286 would respond to extracopy *hetR*, which in the wild type causes constitutive heterocyst formation in nitrogen-replete conditions and production of supernumerary heterocysts with aberrant pattern in medium lacking combined nitrogen (Fig. 1B). Introduction of pWB216S2.4 (Nm^r) (5) carrying wild-type *hetR* into AMC1286

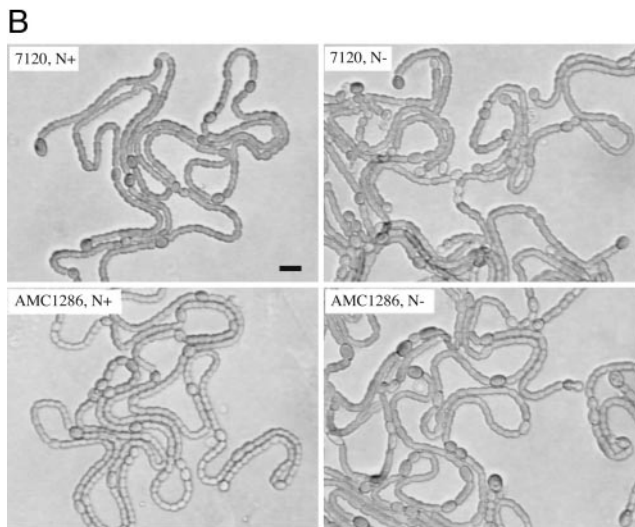
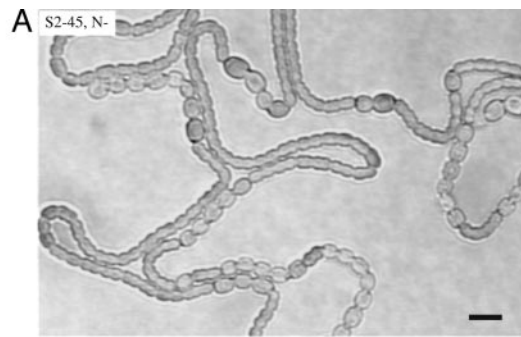


Fig. 1. Pattern-formation defects of a *patS*-overexpression-bypass mutation complemented by *hetR*. (A) Original S2-45 mutant strain containing pAM1888 showing Pat Mch phenotype when grown on combined nitrogen-free BG-11₀ plate. Heterocysts are the larger cells with thicker cell envelopes; cyanophycin granules can be seen at the poles of some heterocysts. (B) *Anabaena* PCC 7120 and AMC1286 strains, both containing extracopy *hetR* supplied in trans on replicative plasmid pWB216S2.4, were grown on BG-11 (N+) or BG-11₀ (N-) plates. (Scale bars, 10 μ m.)

resulted in formation of constitutive heterocysts but with normalized pattern, approximately the same as the wild-type strain carrying the same plasmid (Fig. 1B). This apparent complementation of the Pat Mch phenotype hinted that a recessive mutation in *hetR* might be the cause of the original phenotype. Indeed, PCR amplification and sequencing of *hetR* from strain AMC1285 revealed a single C to T transition at position 667, producing a R223W mutation. To introduce this mutation into the wild-type background, the mutant *hetRR223W* lacking its upstream regulatory region was cloned into the suicide vector pAM2178, and the resulting pAM3316 was integrated into the wild-type *hetR* locus by homologous recombination, producing the merodiploid AMC1287. In this strain, the mutant copy of *hetR* is under its native regulation, whereas the wild-type copy lacks upstream promoter regions and is presumably silent. The reconstructed merodiploid AMC1287 exhibited the same Pat Mch phenotype as the original S2-45 mutant (for example, see Fig. 2).

***hetRR223W* Mutation Overcomes *patS*- and *hetN*-Mediated Negative Regulation of Differentiation.** Introduction of plasmids that overexpress *patS* did not suppress heterocyst formation in AMC1286 and reconstructed AMC1287 merodiploid strains, indicating that both the bypass and Pat Mch phenotypes were caused by the R223W mutation in HetR. The insensitivity to PatS inhibition

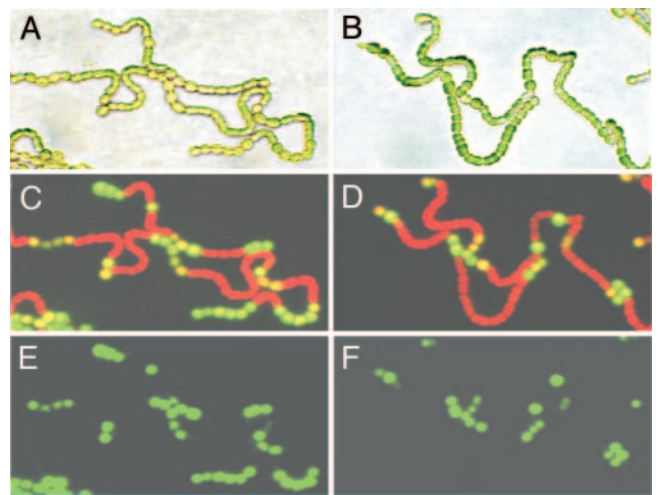


Fig. 2. The R223W mutation in HetR does not inhibit *patS* expression upon nitrogen step-down. The AMC1287 merodiploid strain bearing pAM1951, which carries a partial *patS* ORF followed by *gfp* as a transcriptional reporter (A, C, and E), or pAM1830, which carries a complete *patS* gene followed by *gfp* as a transcriptional reporter (B, D, and F). Images were taken in visible light (A and B) and with fluorescein isothiocyanate-specific illumination (484 ± 8 nm) with (E and F) and without (C and D) GFP-specific emission (518 ± 13 nm) filter sets to cut out red chlorophyll fluorescence from vegetative cells. GFP fluorescence is confined to developing and mature heterocysts.

could result from a lack of response to PatS-mediated signaling or from impaired regulation of *patS* expression. To discriminate between these possibilities, two plasmids were used, pAM1951 containing a *gfp* transcriptional reporter fused to an incomplete *patS* ORF (12) and pAM1830 with the same reporter fused 3' of full-length, active *patS*. Both constructs contain the same ≈ 650 -bp 5' regulatory region.

In the wild-type strain grown diazotrophically, the *gfp* reporter on pAM1951 is expressed only in mature and developing heterocysts, and practically no fluorescence can be detected with the *gfp* reporter on pAM1830, because the overproduction of PatS effectively shuts off its own expression. When introduced into the AMC1287 merodiploid, *gfp* reporters on both pAM1951 and pAM1830 plasmids produced the same pattern of *gfp* expression confined to mature and developing heterocysts (Fig. 2). These data show that the mutant HetR_{R223W} does not impair spatially localized *patS* transcription.

Consistent with the *gfp* reporter data, synthetic PatS-5 pentapeptide (RGSGR), which at 2 μ M completely suppresses differentiation in the wild-type strain (11), did not suppress heterocyst production and did not normalize pattern in the original S2-45 mutant or AMC1287 merodiploid strains (data not shown). It is clear that insensitivity of the mutant HetR_{R223W} to PatS inhibition results from altered interactions in a PatS signaling pathway.

hetN overexpression prevents heterocyst formation in the wild-type strain (13, 14). However, *hetN*-overexpressing plasmid pAM1537 failed to suppress heterocyst formation in the reconstructed merodiploid strain AMC1287 on BG-11₀ plates, whereas it completely inhibited differentiation of the wild type (Fig. 3). Therefore, the mutant HetR_{R223W} protein also has diminished sensitivity to inhibition by HetN.

Ectopic Overexpression of the *hetRR223W* Allele Results in Complete Differentiation. Our initial attempts to reconstruct the *hetRR223W* mutation in the wild-type strain by single recombination with the promoterless mutant allele cloned into a suicide vector produced single recombinants with varying phenotypes,

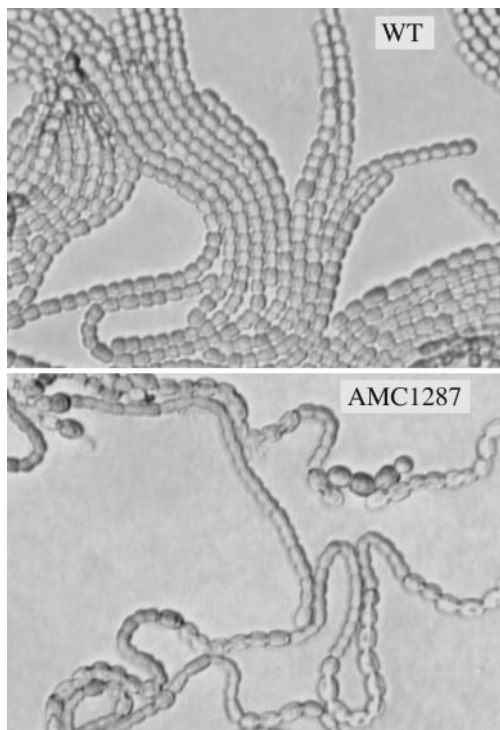


Fig. 3. Extracopy *hetN* fails to suppress heterocyst differentiation in a strain carrying the *hetRR223W* allele. Wild-type *Anabaena* PCC 7120 (A) and the reconstructed merodiploid AMC1287 (B) strains bearing plasmid pAM1537, which carries *hetN* under the control of the copper-inducible *petE* promoter, 2 days after transfer to combined nitrogen-free BG-11₀ plates. The copper concentration in standard BG-11 and BG-11₀ medium should produce approximately half-maximal expression from the *petE* promoter (8).

depending on the nature of the suicide vector used. On BG-11₀ medium, most initial colonies of pAM3316-derived single recombinants had a Pat Mch phenotype very similar to the original S2-45 mutant strain, whereas a few exhibited a wild-type phenotype or contained a mixture of Pat Mch, wild-type, and nearly completely differentiated filaments. When pAM3317 was used for reconstruction, which differs from pAM3316 only in the antibiotic-resistant cassette (Cm^r Em^r C.CE3 versus Sp^r / Sm^r Ω in the same location, see ref. 25) and orientation of the insert, the resultant single recombinants were either similar to the wild type or exhibited a conditionally lethal phenotype, differentiating completely upon transfer to combined nitrogen-free medium (Fig. 4). Heterocyst differentiation was not synchronous, and it took 2–3 days to get nearly 100% heterocysts. One such clone, AMC1288, was used to recover integrated plasmids that contained either the upstream or downstream copies of *hetR*, and DNA sequencing showed that both copies contained the R223W mutation. In another clone with a normal heterocyst pattern, both copies of *hetR* were found to be wild type. Thus, gene conversion leading to two copies of the same allele apparently occurred frequently. High frequency of putative gene conversion between chromosomal and replicative plasmid-borne *hetR* alleles has been observed (5). We suspect that ectopic vector-derived transcription of the downstream copy of *hetRR223W*, which lacks its native promoter region, results in the conditionally lethal phenotype. A similar phenotype with nearly 100% differentiation was observed when strain AMC1289 (see below), containing a *hetRR223W* allele expressed from the copper-inducible *petE* promoter on pAM3318, was incubated in BG-11₀ medium containing 2 μ M copper. The wild-type strain containing this plasmid showed a Mch phenotype but contained strings

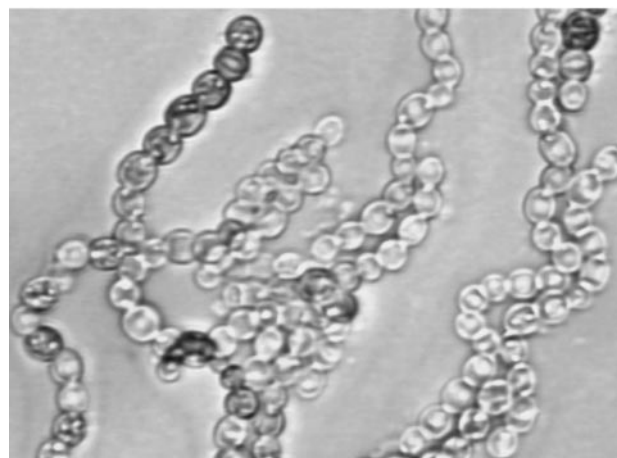
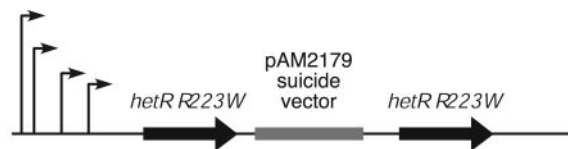


Fig. 4. The strain AMC1288, which contains two copies of the *hetRR223W* allele, showed nearly complete differentiation of vegetative cells into heterocysts 72 h after nitrogen step-down. The map shows the positions of four putative *hetR* transcriptional start sites (8), as indicated by bent arrows. The downstream copy of *hetRR223W* lacks this 5' regulatory region, but may be expressed from upstream vector sequences.

of vegetative cells and could grow diazotrophically, which, together with our earlier complementation experiments, indicates that the wild-type and R223W *hetR* alleles show incomplete dominance.

The conditionally lethal mutant AMC1288, which contains two copies of *hetRR223W*, was used to reconstruct a single-copy *hetRR223W* mutation through looping out of the suicide vector along with one copy of mutant *hetR*. After subculturing AMC1288 in nonselective conditions and streaking on a BG-11₀ plate without antibiotics, several green colonies appeared on the bleached background of completely differentiated filaments. All of these clones had lost the Em^r Cm^r markers of the suicide vector and exhibited the same Pat Mch phenotype as the original S2-45 mutant. One of these clones, designated AMC1289, was used for further phenotypic characterization.

Initial Heterocyst Pattern in the *hetRR223W* Mutant Appears Nearly Random. A striking feature of *hetRR223W* mutants is the formation of irregularly spaced strings of contiguous heterocysts, a phenotype resembling the *de novo* heterocyst pattern of a *patS* mutant, but more extreme. Fig. 5A shows the heterocyst pattern in the wild type 30 and 50 h after nitrogen step-down. The frequency distribution of contiguous vegetative cells peaked at 8 cells at 30 h, and the peak shifted to 10 cells at 50 h; heterocyst frequencies were 10.4% and 7.9%, respectively. The heterocyst pattern in AMC1289 appears to be nearly random, showing a continuous decline in the frequency of contiguous vegetative cells (Fig. 5B). A similar distribution was observed for the number of contiguous heterocysts. The strong bias toward even-numbered intervals of vegetative cells is expected if, during formation of mature heterocysts, the nondifferentiating vegetative cells divide synchronously at least once before the time of observation. Heterocyst frequencies declined from 33.8% at 48 h to 20.5% at 144 h after removal of combined nitrogen, and there was a clear increase in the probability of longer distances

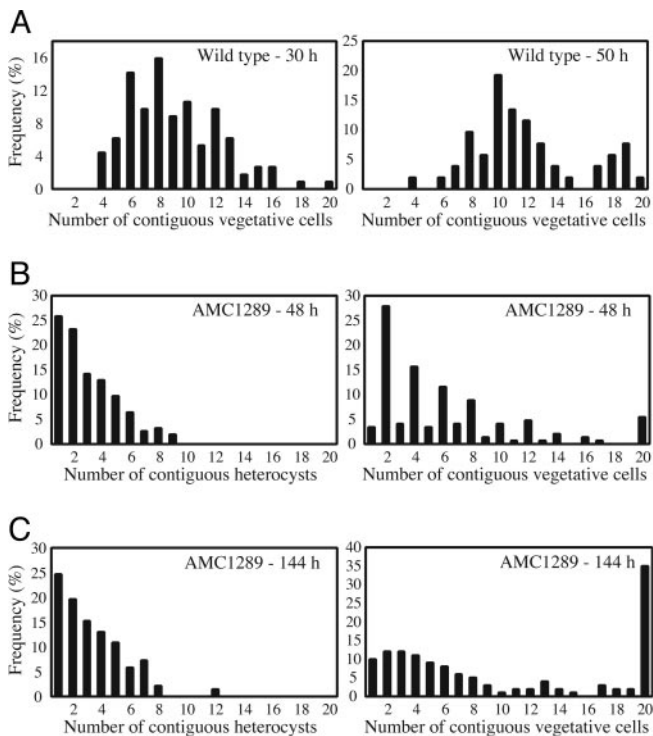


Fig. 5. Heterocyst pattern formation and maintenance in the wild type (A) and AMC1289 *hetRR223W* mutant (B and C). Both strains were grown in BG-11 medium to an OD₇₅₀ of 0.2 and induced to form heterocysts by washing and transfer to BG-11₀ medium. Vegetative cells and heterocysts were scored microscopically at indicated times after heterocyst induction. The wild type produced <5% heterocyst doublets and no higher numbers of contiguous heterocysts. Interval lengths ≥ 20 are shown as 20. The data shown are representative of two independent experiments.

between clusters of heterocysts after prolonged diazotrophic growth (144 h), although very short distances (two to four cells) still prevailed (Fig. 5C). The distribution of contiguous heterocysts did not change (Fig. 5C).

Discussion

When grown without combined nitrogen, $\approx 8\text{--}10\%$ of *Anabaena* PCC 7120 vegetative cells differentiate at semiregular intervals along a filament into highly specialized nitrogen-fixing heterocysts. A mechanism to establish control over heterocyst frequency and spacing is required to ensure the efficient supply of fixed nitrogen to vegetative cells and fixed carbon, as a source of energy and reductant, from vegetative cells to heterocysts. The molecular mechanisms accounting for such control are poorly understood. Among several known genes that influence the heterocyst pattern, the *patS* gene, which encodes a small peptide, is an essential negative regulator of pattern formation (11). To identify components of the PatS-signaling pathway, we used transposon mutagenesis to isolate bypass mutants of *patS*-overexpressing strains. However, this experimental design mainly yielded mutant strains in which the gene of interest was not tagged. The transposons had inserted into genes nonessential for diazotrophic growth, and secondary mutations were apparently responsible for the bypass phenotype.

Because the majority of these bypass mutants exhibited a normal heterocyst pattern, which would be expected if the mutations effected the expression or copy number of the plasmid-borne *patS*, we concentrated on rare mutants that showed an abnormal heterocyst pattern because these were more likely to be impaired in components of the *patS* signaling pathway. The

investigation of one such mutant, S2-45, revealed that the Pat Mch phenotype was caused by a mutation in the *hetR* gene.

The HetR protein has long been known to play a key role as an activator of heterocyst differentiation, but its role in pattern formation was less clear. Our data provide genetic evidence that HetR plays a central role not only in controlling the differentiation process, but also in processing heterocyst pattern formation signals, and that these functions can be separated by mutation. Reconstruction of the *hetRR223W* mutation in the wild-type background allowed us to show unambiguously that a single mutation is responsible for resistance to both known negative regulators of differentiation, PatS and HetN. A striking consequence of the *hetRR223W* mutation is an apparently random pattern of heterocysts along filaments. Thus, this *hetR* allele appears to be insensitive to the major signals governing initial heterocyst pattern formation.

The simplest explanation for the loss of resistance to PatS inhibition in our mutant is that HetR is a direct target of the PatS gene product. Recent results from Zhao and coworkers (10) show that this is very likely to be the case, because the PatS-5 pentapeptide inhibited HetR DNA-binding activity in mobility-shift assays (10). Simultaneous acquisition of resistance to inhibition by overexpression of *patS* and *hetN* indicates that both negative regulators work through HetR, which then acts as the central regulator of heterocyst pattern by determining which cells differentiate. However, PatS and HetN signaling pathways are apparently independent upstream of HetR because they show their strongest influence on different stages of pattern formation and they do not need each other to exert their inhibitory effect on differentiation, because a *patS* mutant is sensitive to inhibition by overexpressed *hetN*, and vice versa (S. Callahan, personal communication; I.Y.K., unpublished data). HetN apparently does not play a role in *de novo* pattern formation, because a strain with the chromosomal *hetN* gene under the control of the copper-regulated *petE* promoter in copper-free medium differentiates single heterocysts with normal spacing in the first round of differentiation upon nitrogen step-down (15). Only subsequent rounds of differentiation produce semiregularly spaced stretches of contiguous heterocysts. The same sequence of events was observed in the *hetN* mutant strain DR994a (14) after nitrogen step-down (I.Y.K., unpublished data). The *de novo* pattern in a *patS* mutant, although strongly disturbed, is not completely random and normalizes partially during diazotrophic growth (12). The phenotype of *hetRR223W* mutant strains could result from the inability to respond to the two different patterning signals that are normally superimposed on each other. It is not known whether PatS and HetN signaling, apart from the supply of fixed nitrogen (12), are the only pathways that regulate the heterocyst pattern. It would be interesting to determine whether the phenotype of a *patS hetN* double mutant mirrors the phenotype of the R223W mutation in HetR.

Ectopic overexpression of the *hetRR223W* allele in a *hetRR223W* background results in almost complete differentiation and a conditionally lethal phenotype in combined nitrogen-free medium. It is well known that a minimal filament length is required to allow heterocyst differentiation to proceed. Two explanations have been suggested for this phenomenon (33). The first is a nutritional one and implies a dependence of differentiating cells on photosynthate supplied by neighboring vegetative cells. An alternative explanation postulates that a minimum number of vegetative cells are required to inactivate a diffusible morphogen that will otherwise accumulate in presumptive heterocyst and inhibit differentiation. The ability of strain AMC1288 to differentiate almost 100% morphologically distinct heterocysts upon nitrogen step-down indicates a great degree of metabolic autonomy of differentiating heterocysts and makes intercellular signaling the more plausible explanation.

The PatS peptide, or a product of its cleavage, has been hypothesized to function as a diffusible morphogen (11). Because the localized induction of *patS* and *hetR* expression occurs in the same cells, HetR must not be inhibited by PatS in cells that successfully differentiate. One can envisage several ways in which HetR could avoid this inhibition. First, spatially localized induction of HetR expression probably precedes induction of PatS expression because *patS* transcription is HetR-dependent. No induction of a *patS-gfp* reporter (unpublished data) and no accumulation of *patS* message (10) occur in a *hetR* mutant. This could result in a high HetR/PatS ratio in differentiating cells. Second, *patS* message could be degraded rapidly or its translation blocked, or PatS could be inactivated in proheterocysts by a protease, an inhibitor, or through posttranslational modification. Third, posttranslational modification of HetR itself could render it insensitive to inhibition. Fourth, assuming a model in which PatS is a diffusible morphogen, PatS could be efficiently exported only from differentiating cells. However, there is currently no proof that PatS acts as a morphogen, which leaves the question of apparent PatS-mediated intercellular signaling open to alternative explanations. However, the formation of a normal pattern must depend on the sensitivity of HetR to PatS inhibition at early stages of differentiation in those cells that remain vegetative cells. The essentially random initial pattern of heterocysts produced by a *hetRR223W* mutant shows that these cells have lost normal sensitivity to the PatS inhibitory pathway.

In most heterocystous cyanobacteria, the differentiation process is controlled by the availability of external combined nitrogen. At first glance, the *hetRR223W* mutant behaves normally in this respect because it does not produce heterocysts constitutively on media with combined nitrogen. This is different from a *patS* mutant, which forms a few percent heterocysts in a medium containing nitrate (11, 12). However, the *hetRR223W* mutant appears to be altered in its sensitivity to nitrogen. Differentiation of this strain is sluggish, lagging behind the wild

type by $\approx 8\text{--}12$ h in liquid; in very dilute suspensions, it is inhibited at nitrate concentrations of 0.05 mM, approximately five times lower than in the wild type (unpublished data). In *hetRR223W* mutants, *hetR* expression appears to be activated and differentiation initiated only after filaments have exhausted extracellular and intracellular supplies of available nitrogen and a severe shift in C/N balance is perceived by the cells. This could explain the delay in development and why the bleaching of filaments before formation of functional heterocysts is far more pronounced in the *hetRR223W* mutant than in the wild type. Our preliminary data suggest that, unlike the wild type, in the *hetRR223W* mutant, the signal that induces differentiation is not a sudden step-down or gradual decrease below a threshold level of exogenous combined nitrogen, but the perception of nitrogen starvation by an intracellular sensor. These data might fit into a recently proposed model in which external combined nitrogen entering cells is recognized differently from fixed nitrogen produced by heterocysts, and the signal controlling differentiation is related to external availability of fixed nitrogen but not to the nitrogen sufficiency of individual cells (34). This model implies that the wild type employs a strategy that anticipates nitrogen starvation, and the decision to produce heterocysts is made before the filaments begin to experience actual nitrogen limitation. The benefits of this strategy are evident.

The most recent experimental evidence that HetR is a DNA-binding protein and that PatS-5 pentapeptide inhibits its DNA-binding activity (10) provides the first mechanistic view of HetR function and regulation. Our genetic data complement these findings and provide insights into the complex regulation of HetR activity. The ability to construct conditionally lethal strains that undergo complete differentiation after nitrogen step-down presents a powerful tool for screening for mutants specifically impaired in early stages of differentiation.

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