

## HetF and PatA Control Levels of HetR in *Anabaena* sp. Strain PCC 7120<sup>∇</sup>

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***Anabaena* sp. strain PCC 7120 is a filamentous cyanobacterium that differentiates heterocysts in response to deprivation of combined nitrogen. A *hetF* deletion strain lacked heterocysts and had aberrant cell morphology. Site-directed mutagenesis of the predicted active-site histidine and cysteine residues of this putative caspase-hemoglobinase fold protease abolished HetF function, supporting the hypothesis that HetF is a protease. Deletion of *patA*, which is necessary for the formation of most intercalary heterocysts, or *hetF* resulted in an increase in HetR protein, and extra copies of *hetF* on a plasmid functionally bypassed the deletion of *patA*. A *hetR-gfp* translational fusion expressed from an inducible promoter demonstrated that *hetF*-dependent down-regulation of HetR levels occurs rapidly in vegetative cells, as well as developing heterocysts. “Mosaic” filaments in which only one cell of a filament had a copy of *hetR* or *hetF* indicated that *hetF* is required for differentiation only in cells that will become heterocysts. *hetF* was required for transcription from a *hetR*-dependent transcription start point of the *hetR* promoter and induction of transcription from the *patS* promoter. The inverse correlation between the level of HetR protein and transcription from *hetR*-dependent promoters suggests that the transcriptional activity of HetR is regulated by HetF and PatA.**

*Anabaena* sp. strain PCC 7120 is a filamentous cyanobacterium capable of reducing atmospheric dinitrogen in specialized cells known as heterocysts, which provide the microaerophilic environment required for nitrogen fixation by the O<sub>2</sub>-labile nitrogenase enzyme complex. When sources of fixed nitrogen are scarce, approximately 10% of the cells in filaments differentiate into heterocysts to form a regular pattern (24). This simple, one-dimensional pattern of two interdependent cell types makes heterocyst formation in *Anabaena* sp. strain PCC 7120 one of the paradigm systems for the regulation of cellular differentiation and pattern formation in bacteria. Although many genes regulating the process of heterocyst formation have been discovered (11, 30), a clear model of the genetic network regulating this process is still emerging.

The master regulator of heterocyst formation is thought to be HetR. A *hetR* mutant is incapable of differentiating heterocysts, and ectopic overexpression leads to the formation of multiple contiguous heterocysts, even in the presence of nitrate or ammonia, conditions that suppress differentiation by the wild type (4, 5). Therefore, expression of *hetR* is both necessary and sufficient for heterocyst differentiation in a wild-type genetic background. Transcriptional regulation of *hetR* is complex. Four transcription start points (TSPs) have been defined within the *hetR* promoter region (5). The –728 and –696 TSPs are dependent on *ntcA* via *nrrA*, which binds directly to the region of DNA upstream of these two TSPs, while the –271 TSP is dependent on *hetR* itself, an example of positive auto-regulation (7, 18). Transcription from the –184 TSP appears to be constitutive (18). Induction of *hetR* transcription occurs

early in the developmental process and is enhanced in developing heterocysts (2).

HetR has been reported to possess DNA-binding and auto-proteolytic, Ca<sup>2+</sup>-dependent serine-protease activities, both of which appear to be essential for heterocyst formation (13, 33). HetR has been shown to bind to the promoter regions of *patS*, *hepA*, and *hetR* in vitro, and *hetR* is required for transcriptional upregulation of these three genes in vivo (13). Serine residues at positions 152 and 179 of HetR have been reported to be required for protease activity, with the serine at residue 152 reported to be the active site (33). However, a recent study conflicts with the original reports, demonstrating that the serine at position 152 is not required to promote heterocyst formation, suggesting that this may not be the active site of HetR protease activity or that HetR protease activity may not be required for heterocyst differentiation (21). Although the role of HetR protease activity is unclear, proper turnover of HetR appears to correlate with the ability of HetR to promote heterocyst formation, since glycine, histidine, and serine residues at positions 36, 69, and 179, respectively, are required for both normal HetR protein turnover and heterocyst formation (21, 32).

The genes *patS* and *hetN*, which encode negative regulators of heterocyst formation, act at the level of HetR. The *patS*-derived pentapeptide RGSGR is capable of inhibiting HetR DNA-binding activity in vitro and heterocyst formation in vivo (13, 14, 27). Along with *hetR*, *patA* has been shown to positively influence heterocyst formation. However, unlike *hetR*, inactivation of *patA* results in heterocyst formation primarily at the filament termini. Inactivation of *patA* prevents the patterned upregulation of transcription of *hetR* found in the wild type, but ectopic overexpression of *hetR* is insufficient to bypass inactivation of *patA*, suggesting that *patA* affects positive auto-regulation of *hetR* transcription and HetR activity (15). The function of *patA* may be to attenuate *patS*- or *hetN*-dependent

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inhibition of heterocyst differentiation because strains with *patA* and *patS* or *patA*, *patS*, and *hetN* inactivated have a partially restored ability to form intercalary heterocysts (20). The C terminus of PatA is similar in sequence to the response regulator CheY, and the N terminus contains a recently defined PATAN domain, which is predicted to be involved in protein-protein interaction (15, 16). To date, however, no biochemical characterization has elucidated the exact function of PatA.

A third gene that positively regulates heterocyst formation, *hetF*, has been characterized in the closely related species *Nostoc punctiforme* (26). Like *hetR*, *hetF* is essential to heterocyst formation, and the presence of *hetF* on a multicopy plasmid results in the differentiation of clusters of cells to give a multiple contiguous heterocyst phenotype. Induction of *hetR* transcription was delayed and failed to localize to specific cells in a *hetF*-null mutant. Extra copies of *hetR* on a plasmid were insufficient to bypass inactivation of *hetF*, suggesting that, like *patA*, *hetF* affects positive autoregulation of *hetR* transcription and HetR activity. A *hetF* deletion strain carrying extra copies of *hetR* on a plasmid also had an irregular cell morphology, and a functional *hetR-gfp* translational fusion suggested that levels of HetR are higher in the deletion strain than in the wild type (26). It has been suggested that the mechanism of HetF in cellular differentiation may be promotion of cell-specific accumulation of HetR. Since the report of its discovery, HetF has been predicted to contain a caspase-hemoglobinase fold (CHF), the proteolytic domain in a wide range of cysteine-dependent proteases, and to be most closely related to the eukaryotic separases (1). The *hetF* homologue in *Anabaena* sp. strain PCC 7120 has been shown to be necessary for nitrogen fixation (25), but its role in cellular differentiation has not been characterized.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The growth of *Escherichia coli* and *Anabaena* sp. strain PCC 7120 and its derivatives; concentrations of antibiotics; the induction of heterocyst formation in BG-11<sub>0</sub> medium, which lacks a combined-nitrogen source; the regulation of  $P_{petE}$  and  $P_{nir}$  expression; and conditions for photomicroscopy were as previously described (21). Plasmids were conjugated from *E. coli* to *Anabaena* sp. strain PCC 7120 and its derivatives as previously described (8).

**Construction of plasmids created in this study.** The plasmids and strains used in this study are described in Table 1. The primers used in PCR are described in Table 2. All PCR-generated constructs were sequenced to verify their integrity. Plasmid pDR256 was used to delete the *hetF* coding region. A 673-bp region upstream of *hetF* containing the first 9 bp of the *hetF* coding region was amplified from the chromosome (with primers *hetF*-Up-F and *hetF*-Up-R) and cloned into pBluescript SK+ (Stratagene) as a BamHI-KspI fragment by using restriction sites introduced on the primers. An 808-bp region downstream of *hetF* including the last 9 bp of the *hetF* coding region was amplified from the chromosome (with primers *hetF*-Down-F and *hetF*-Down-R) and cloned into this plasmid as a KspI-SacI fragment by using restriction sites introduced on the primers. A BamHI-SacI fragment containing the regions both upstream and downstream of *hetF* was moved into pRL277 (2) at the BglII and SacI sites to create pDR256.

pDR267 is a mobilizable shuttle vector containing the *hetF* promoter and coding region. A 4,763-bp region containing the *hetF* promoter and coding region was amplified from the chromosome (with primers *PhetF*-SacI-F and *hetF*-BamHI-R) and cloned into pAM505 (23) as a SacI-BamHI fragment by using restriction sites introduced on the primers to create pDR267.

pDR282 and pDR284 are mobilizable shuttle vectors containing the *hetF* promoter and coding region with nucleotide substitutions resulting in H201Y and C246A amino acid substitutions, respectively. A 4,763-bp fragment containing the *hetF* promoter and coding frame was amplified from the chromosome (with primers *hetFH201Y*-F and *hetFH201Y*-R or *hetFC246A*-F and

*hetFC246A*-R, along with *hetF*-Up-F and *hetF*-BamHI-R) via overlap extension PCR as previously described (12). The resulting products were cloned into pDR267 as KpnI-BamHI partial fragments to create pDR282 and pDR284.

pDR281 is a mobilizable shuttle vector containing the *hetF* promoter and coding frame with six histidine codons added to the 3' end of the coding frame. A 4,778-bp fragment containing the *hetF* promoter and coding region plus six histidine codons [*hetF*(H6)] was amplified from the chromosome (with primers *hetF*-Up-F and *hetF*-H6-BamHI-R). The resulting product was cloned into pDR267 as a KpnI-BamHI partial fragment to create pDR281.

pDR255 is a pET21a (Novagen) derivative used to overexpress *hetF*(H6) in *E. coli*. A 2,502-bp region containing the *hetF* coding region plus six histidine codons added to the 3' end was amplified from the chromosome (with primers *hetF*-NdeI-F and *hetF*-H6-BamHI-R) and cloned into pET21a as an NdeI-BamHI partial fragment to create pDR255.

pDR204, pDR216, and pDR226 are mobilizable shuttle vectors containing  $P_{nir}$  transcriptionally fused to *hetR*(H6), *hetR*, and *hetR*(S179N), respectively, and were constructed as previously described (21).

pSMC138 is a mobilizable shuttle vector containing a  $P_{petE-gfp}$  transcriptional fusion. The *petE* promoter and *gfp* were amplified from chromosomal DNA and pAM1956, respectively, and fused by overlap extension PCR (with primers *PpetE*-F, *PpetE-gfp*-R, *gfp*-*PpetE*-F, and *gfp*-KPN1-R). The 1,062-bp fragment containing  $P_{petE-gfp}$  was cloned into pGEM-T (Promega) and subsequently moved into pAM505 as a KpnI-SacI fragment to create pSMC138.

pSMC148 is an expression vector used for production of HetR with an N-terminal polyhistidine epitope tag in *E. coli*. The coding region of *hetR* was amplified from chromosomal DNA with primers *hetRcf*-NdeI and *hetRcr*-SacI, cloned into pGEM-T, and subsequently moved to pPROEX-1 (Life Technologies) as an NdeI-SacI fragment to generate pSMC148.

pDR293 is a mobilizable shuttle vector containing the gene for the green fluorescent protein (GFP), *gfp*, translationally fused to the 3' end of *hetR*, the transcription of which is under the control of the copper-inducible *petE* promoter. A 758-bp region containing the *gfp* coding frame and a linker region 5' of the *gfp* start codon was amplified from pAM1956 (with primers TNL-GFP-F and TNL-GFP-R) and cloned into pAM504 (23) as a BamHI-SacI fragment by using restriction sites introduced on the primers to create pSMC232. A 1,239-bp region containing the *petE* promoter transcriptionally fused to the *hetR* coding region was amplified from pDR120 (19) (with primers *PpetE*-BamHI-F and *hetR*-SmaI-R) and cloned into pSMC232 as a BamHI-SmaI fragment with sites introduced on the primers to create pDR293.

pDR309 and pDR310 are mobilizable shuttle vectors containing a  $P_{petE-gfp}$  transcriptional fusion and either a  $P_{nir-hetR}$ (H6) transcriptional fusion or the *hetF* promoter and coding region, respectively. A 1,074-bp region containing a  $P_{petE-gfp}$  transcriptional fusion was amplified from pSMC138 (with primers *PpetE*-F-SacI and TNL-GFP-R) and cloned into pDR204 and pDR267 as a SacI fragment by using restriction sites introduced on the primers to create pDR309 and pDR310, respectively.

pDR311, pDR312, and pDR350 are mobilizable shuttle vectors carrying transcriptional fusions between the reporter gene *lacZ* and the *nir*, *hetF*, and *petE* promoters, respectively. The *lacZ* gene was amplified from *E. coli* strain K-12 with primers *Txp-LacZ*-F and *Txp-LacZ*-R and cloned directly into pAM504 as a BamHI-KpnI fragment with sites on the primers to yield pNC101. The promoter regions of the *nirA* (primers *Pnir*-BamHI-F and *Pnir*-SmaI-R), *hetF* (primers *PhetF*-BamHI-F and *PhetF*-SmaI-R), and *petE* (primers *PpetE*-BamHI-F and *PpetE*-SmaI-R) genes were generated by PCR and cloned directly into pNC101 to make plasmids pDR311, pDR312, and pDR350, respectively.

Plasmids pDR324 and pDR266 are mobilizable shuttle vectors containing transcriptional fusions between the promoter regions of *nirA* or *hetF* and *gfp*, respectively. The promoter region of *nirA* was amplified by PCR with primers *nirI*-SacI and *Pnir*-SmaI-R and cloned directly into pAM1956 (28) as a SacI-SmaI fragment to make pDR324. The promoter region of *hetF* was amplified by PCR with primers *PhetF*-SacI-F and *PhetF*-SmaI-R and cloned directly into pAM1956 as a SacI-SmaI fragment to make pDR266.

**Strain construction.** Deletion of the *hetF* coding region was performed as previously described (3), by using plasmid pDR256 to delete cleanly the *hetF* coding region in *Anabaena* sp. strains PCC 7120, UHM100, UHM101, UHM103, and UHM109 to create strains UHM130, UHM131, UHM135, UHM132, and UHM134, respectively. The resultant strains were screened via colony PCR with primers *PhetF*-Seq-1 and *hetF*-con-R, which anneal outside of the region of DNA used on plasmid pDR256 to make the deletion, and tested for streptomycin and spectinomycin sensitivity.

**Generation of a polyclonal antibody to HetR.** HetR with an N-terminal polyhistidine epitope tag was purified from *E. coli* strain BL21 (Stratagene) carrying plasmid pSMC148 as previously described (21). Five milligrams of protein at a

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>Anabaena</i> sp. strains <sup>a</sup>		
PCC 7120	Wild type	Pasteur Culture Collection
216	<i>hetR(S179N)</i>	4
UHM100	P <sub>petE</sub> - <i>hetN</i> Δ <i>patS</i>	3
UHM101	Δ <i>patA</i>	20
UHM103	Δ <i>hetR</i>	3
UHM109	Δ <i>hetR</i> Δ <i>patA</i>	20
UHM130	Δ <i>hetF</i>	This study
UHM131	P <sub>petE</sub> - <i>hetN</i> Δ <i>patS</i> Δ <i>hetF</i>	This study
UHM132	Δ <i>hetR</i> Δ <i>hetF</i>	This study
UHM134	Δ <i>hetR</i> Δ <i>patA</i> Δ <i>hetF</i>	This study
UHM135	Δ <i>patA</i> Δ <i>hetF</i>	
Plasmids		
pAM504	Shuttle vector for replication in <i>E. coli</i> and <i>Anabaena</i> ; Km <sup>r</sup> Nm <sup>r</sup>	23
pAM505	Same as pAM504 with multiple cloning site inverted	23
pAM1951	pAM505 with P <sub>patS</sub> - <i>gfp</i>	29
pAM1956	pAM505 with promoterless <i>gfp</i>	29
pRL277	Suicide vector; Sm <sup>r</sup> Sp <sup>r</sup>	2
pET21a	Expression vector; Ap <sup>r</sup>	Novagen
pPROEX-1	Expression vector for making polyhistidine epitope-tagged proteins; Ap <sup>r</sup>	Life Technologies
pSMC127	pAM504 carrying P <sub>hetR</sub> - <i>gfp</i>	6
pSMC138	pAM505 carrying P <sub>petE</sub> - <i>gfp</i>	This study
pSMC148	pPROEX-1 carrying <i>hetR</i>	This study
pAM1951	Shuttle vector carrying P <sub>patS</sub> - <i>gfp</i>	28
pDR204	pAM505 carrying P <sub>nir</sub> - <i>hetRH6</i>	21
pDR216	pAM505 P <sub>nir</sub> - <i>hetR</i>	21
pDR226	pAM505 P <sub>nir</sub> - <i>hetRH6(S179N)</i>	21
pDR255	pET21a carrying <i>hetFH6</i>	This study
pDR256	Suicide plasmid based on pRL277 used to delete <i>hetF</i>	This study
pDR266	pAM1956 with P <sub>hetF</sub> fused to <i>gfp</i>	This study
pDR267	pAM505 carrying P <sub>hetF</sub> - <i>hetF</i>	This study
pDR281	pAM505 carrying P <sub>hetF</sub> - <i>hetFH6</i>	This study
pDR282	pAM505 carrying P <sub>hetF</sub> - <i>hetF</i> (H201Y)	This study
pDR284	pAM505 carrying P <sub>hetF</sub> - <i>hetF</i> (C246A)	This study
pDR293	pAM504 P <sub>petE</sub> - <i>hetR-gfp</i>	This study
pDR302	pAM505 carrying P <sub>hetF</sub> - <i>hetF</i> and P <sub>nir</sub> - <i>hetRH6</i>	This study
pDR309	pAM505 carrying P <sub>nir</sub> - <i>hetRH6</i> and P <sub>petE</sub> - <i>gfp</i>	This study
pDR310	pAM505 carrying P <sub>hetF</sub> - <i>hetF</i> and P <sub>petE</sub> - <i>gfp</i>	This study
pDR311	pAM504 carrying P <sub>nir</sub> - <i>lacZ</i>	This study
pDR312	pAM504 carrying P <sub>hetF</sub> - <i>lacZ</i>	This study
pDR324	pAM1956 with P <sub>nir</sub> fused to <i>gfp</i>	This study
pDR350	pAM504 carrying P <sub>petE</sub> - <i>lacZ</i>	This study
pPetHetR	Shuttle vector carrying P <sub>petE</sub> - <i>hetR</i>	5

<sup>a</sup> For the *Anabaena* strains constructed in this study, mutations were introduced in the order indicated.

concentration of 1 mg ml<sup>-1</sup> was digested with tobacco etch virus protease according to the manufacturer's (Invitrogen) specifications to remove the epitope tag and subsequently dialyzed against TSEG buffer (21), incubated with 1 ml Ni-nitrilotriacetic acid agarose (Qiagen) for 1 h at 4°C, and added to a column, and the eluate was collected to separate it from the tobacco etch virus protease, which has the epitope tag, and the epitope tag was cleaved from the HetR protein. The resulting protein, which is predicted to be wild-type HetR with the addition of residues GAH preceding the N-terminal methionine, was sent to New England Peptide (Gardner, MA) for the generation of a polyclonal antibody in rabbits.

**Western blot analysis.** Two milliliters of cultures collected at the indicated time points was centrifuged at 2,000 × g for 3 min, resuspended in 8% sodium dodecyl sulfate (SDS), incubated at 100°C for 10 min, and centrifuged at 13,000 × g for 10 min to remove insoluble cell debris. Protein was precipitated from the supernatant by the addition of acetone to 80% and centrifuged at 13,000 × g for 10 min. Pellets were air dried and dissolved in a final volume of 25 μl 8% SDS. Protein concentrations were determined by the DC Protein Assay (Bio-Rad Laboratories). A 3.5-μg sample of protein was then subjected to SDS-polyacrylamide gel electrophoresis and electrophoretic transfer onto polyvinylidene difluoride membrane. HetR and HetF were detected with Penta-His

antibodies (Qiagen) or a polyclonal antibody directed against HetR, followed by chemiluminescence detection (Western Breeze; Invitrogen). Detection of Penta-His antibodies was performed by exposure to Lumi-Film Chemiluminescent Detection Film (Roche), and detection of polyclonal anti-HetR antibodies was performed with the GeneGnome BioImaging System (Syngene). Coomassie staining of the membrane was performed with Simply Blue Safe Stain (Invitrogen). All Western blot analyses were performed in duplicate.

**β-Galactosidase assays.** Cells for β-galactosidase assays were treated as previously described (22), except that in place of treatment with chloroform, cavitation for 4 min in a Branson 1510 cavitator was used to lyse cells. After centrifugation to remove cell debris, β-galactosidase activity of the aqueous phase was measured according to Miller (17) and protein concentration was measured with the DC Protein Assay. Activity was expressed as nanomoles of *o*-nitrophenol per minute per milligram of protein.

**Rapid amplification of cDNA ends (RACE).** One-hundred-milliliter cultures were harvested 18 h after removal of combined nitrogen by centrifugation at 2,000 × g, and the cells were frozen in a liquid nitrogen bath. Frozen cell pellets were ground with a mortar and pestle while mixed with liquid nitrogen for 45 min and then mixed with 5 ml TRIzol (Invitrogen) and incubated at room temperature for 1 h. The mixture was then centrifuged at 10,000 × g for 10 min at 4°C.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide <sup>a</sup>	Sequence
hetF-NdeI-F	CATATGAATGCCCATTTGAAATCAG
hetF-H6-BamHI-R	ATATAGGATCCCTAGTGTGGTATGGTAT GCTTGGGGCTTTTTGTG
hetF-Up-F	ATATAGGATCCAGTCGGTGTAGCCAGCTAG
hetF-Up-R	GATTTCCGCGGGTCATTCATCAATGCCAG
hetF-Down-F	AACAACCGCGGCCAAGTAGAGTACGGGAAG
hetF-Down-R	TTGCAGAGCTCTGACCTAAAGCCGCCATTTTC
hetF-BamHI-R	ATATAGGATCCCTACTTGGGGCTTTTTGTG
hetFH201Y-F	CTACTCGGTTATAGCAATTTAGGCGCGAACG
hetFH201Y-R	CTAAATTTGCTATAACCGGAGTAGTGTAAAA CATG
hetFC246A-F	GTTAACTCCGCGTGGGGTACCTACACCG CGAG
hetFC246A-R	GGTACCCACGCGGAGTAAACACCGCCA TTTG
TNL-GFP-F	TTTGGATCCAATCCCGGGGATCGGCGTCAGCT AGTAAAGGAGAAGAACTTTTCACTGGA
TNL-GFP-R	ACAGAGCTCTATTGTATAGTTTCATCCATGC CATG
PhetF-SacI-F	ATATAGAGCTCTGTAGCTCTGTGTCTCTT GTTG
PhetF-SmaI-R	ATATACCGGGCCGCCAACCAATCAGCTAC
PpetE-BamHI-F	ATATAGGATCCCTGAGTACTGAGTACACAG
PpetE-SmaI-R	ATATACCGGGACCTGTAGTTTATTTTCTTA TTTC
Pnir-SmaI-R	ATATACCGGGAAGTTTTTTTGTCTCAAGATCA ATCC
Pnir-BamHI-F	GCGCGGGATCCAGCTACTCATTAGTTAAGTG TAATG
PhetF-BamHI-F	ATATAGGATCCTGTAGCTCTGTGTCTCTTG
hetR-SmaI-R	ATATACCGGGAATCTCTTTTCTACCAA CACC
nir1-SacI	GCGCGGAGCTCAGCTACTCATTAGTTAAGTG TAATG
hetRcf-NdeI	CATATGAGTAACGACATCGATCTGATC
hetR6H-r	TTAGTGATGGTATGGTATGATCTTCTTTTC TACCAAACACCATTTG
hetRH6-SacI-R	TTACTGAGCTCTTAGTGATGGTATGGTATGAT ATCTTCTTTTCTACCAAACACCATTTG
hetRcr-SacI	GAGCTCTTAATCTCTTTTCTACCAAACACC
PpetEF-SacI	TTTGGATCTGCAGTCGGTAATTTGTTGTTT TTCC
PhetF-Seq-1	AAATTGCATCAAAGGTATGCTG
hetF-con-R	GTATGGACAAGATTACCAGC
696newII	AATCGACAATCTCTCCTTGAGGTG
184newI	CATTGCACTGGGGCCAAGACGCTT
696newI	TTTGCTTATGGCAGTGTAGGTCG
New184nesrev	AAAGTAGTTGGTATACACCCATAGGGGG
PpetE-F	GCTGAGGACTGAGTACACAGCTAAT
PpetE-gfp-R	TTCTCCTTACTCATGGCGTTCTCTAACCT GTAG
Txp-LacZ-F	CGCGGATCCATCCCGGGAGGAAACAGCTATG ACCATTAC
Txp-LacZ-R	CGCGGTACTTATTTTTGACACCAGACCAA CTGG
gfp-PpetE-F	TAGGAGAACGCATGAGTAAAGGAGAAGAAC TTTTCACTG
gfpKPN1-R	GGTACCTATTTGTACAATTCATCCATACC

<sup>a</sup> Oligonucleotides are shown in the 5'-to-3' direction.

One milliliter of chloroform was added to the supernatant, and the mixture was shaken, incubated for 15 min at room temperature, and centrifuged at 10,000 × g for 10 min at 4°C. RNA was precipitated from the supernatant by the addition of 2.5 ml isopropanol and incubated overnight at -20°C. The RNA was collected by centrifugation at 10,000 × g for 10 min at 4°C, washed with 5 ml of 75% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. The RNA was digested with RNase-free DNase I (Roche) with the addition of Protector RNase Inhibitor (Roche) for 3 h at 37°C. An acid phenol-chloroform extraction was performed, and the RNA was precipitated with 3 M sodium acetate and isopropanol, washed with 70% ethanol, and dried (ToTALLY RNA; Ambion Inc.). The pellet was resuspended in diethyl pyrocarbonate-treated water, and 5 µg of total RNA was used for the RACE procedure according to the 5'/3' RACE kit, 2nd generation (Roche), with primers 696newII and 184newI for synthesis of cDNA for the -728 and -696 and the -271 and -184 transcriptional start sites, respectively. Primers 696newI and New184nesrev were used for amplification of cDNA from the -728 and -696 and the -271 and -184 start

sites, respectively. Products were analyzed with a 3% agarose gel. For all RNA preparations, PCRs with primers hetRcf-NdeI and hetR6H-r were done as negative controls to detect contaminating DNA.

**Creation of "mosaic" filaments.** To create and observe mosaic filaments in which only one cell contained a plasmid, modifications were made to the standard conjugation protocol (8). *E. coli* cells were grown to an  $A_{600}$  of 0.5, washed twice with BG-11, and used either concentrated 30-fold, concentrated 3-fold, diluted 3-fold, or diluted 30-fold. *E. coli* aliquots of 150 µl were combined with 150 µl of *Anabaena* at an  $A_{750}$  of 0.6 and spread onto 40 ml of solid BG-11 medium containing 5% LB by volume in a petri dish (15 by 100 mm). After 24 h, cells were resuspended in 2 ml BG-11, washed twice in BG-11 with centrifugation at 2,000 × g to collect primarily *Anabaena* cells, and resuspended in 2 ml of BG-11 for filaments with the plasmid containing *hetR*, pDR309, or 2 ml of BG-11<sub>0</sub> for filaments with the plasmid containing *hetF*, pDR310. Filaments were examined at this point for GFP fluorescence in isolated cells and the presence of heterocysts. The remainder of the filaments were incubated under standard growth conditions for an additional 24 h before microscopic examination. To induce expression of *gfp* from the *petE* promoter, 3 µM copper was added to all culture media.

Typically, the conjugations with *E. coli* cultures concentrated threefold or diluted threefold yielded many filaments with a single fluorescent cell. Conjugations with *E. coli* cultures concentrated 30-fold yielded filaments with multiple fluorescent cells per filament, occasionally as many as 50% of the cells, and the 30-fold dilution yielded very few filaments with fluorescent cells. For strains that formed heterocysts in response to the plasmid, filaments with a single fluorescing heterocyst were common, presumably because heterocyst formation precluded further cell division. In contrast, strains that did not form heterocysts had primarily multiple contiguous fluorescent vegetative cells, with isolated fluorescent cells representing less than 10% of the total of fluorescing cells.

## RESULTS

**hetF from *Anabaena* sp. strain PCC 7120 is similar to *hetF* from *N. punctiforme*.** To determine if *hetF* from *Anabaena* sp. strain PCC 7120, designated open reading frame all1730, is functionally equivalent to that of *N. punctiforme*, the *hetF* coding region was deleted cleanly from the chromosome of the wild-type strain, *Anabaena* sp. strain PCC 7120. Compared to the wild type, *hetF* deletion strain UHM130 grew poorly in BG-11 and BG-11<sub>0</sub> liquid media, displayed an aberrant cell morphology characterized by enlarged and elongated cells in both BG-11 and BG-11<sub>0</sub> (the latter lacks a source of fixed nitrogen), and was unable to form heterocysts even after several days in BG-11<sub>0</sub> (Fig. 1A and B). When the *hetF* promoter and coding region were introduced on replicating plasmid pDR267 to complement UHM130, the aberrant cell morphology was resolved and multiple contiguous heterocysts were observed 24 h after nitrogen step-down and thereafter (Fig. 1C). UHM130 complemented with *hetF* on a plasmid did not form extra heterocysts in BG-11, which contains nitrate, and vegetative filaments were slightly irregular (data not shown). Cells were also significantly smaller than those of the wild type in both BG-11 and BG-11<sub>0</sub>. When a fusion between the promoter of *hetF* and *gfp* was introduced on plasmid pDR266 into the wild-type strain, fluorescence appeared moderate and unpatterned and showed no noticeable induction upon nitrogen step-down (data not shown). Several aspects of *hetF* from *N. punctiforme* and *Anabaena* sp. strain PCC 7120 appear to be very similar (26).

**Conserved histidine and cysteine residues required for HetF activity.** Since the discovery of HetF, a bioinformatic study indicated that a region of HetF is homologous to the CHF class of cysteine proteases and is most closely related to the eukaryotic separases (1), but to date this relationship has not been demonstrated functionally. As a first step toward determining

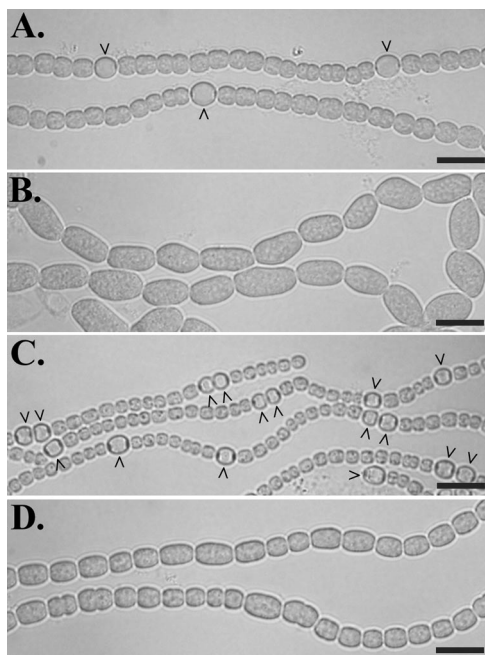


FIG. 1. Phenotype of a *hetF* deletion strain, UHM130, 48 h after the removal of combined nitrogen. *Anabaena* sp. strain PCC 7120 (A); *hetF* deletion strain UHM130 (B); UHM130 complemented with pDR267, a plasmid bearing  $P_{hetF}$ -*hetF* (C); and UHM130 carrying pDR282, a plasmid bearing  $P_{hetF}$ -*hetF*(H201Y) (D), are shown. Carets indicate heterocysts. Bars = 10  $\mu$ m.

if HetF is indeed a protease, sequence alignments were used to predict the active-site cysteine and histidine residues of HetF that correspond to the catalytic dyad of known CHF proteases (data not shown), and changes to conservative substitutions with alanine and tyrosine, respectively, were made in *hetF*. Plasmids pDR282 and pDR284, bearing *hetF* with H201Y and C246A substitutions, respectively, failed to complement the heterocyst deficiency of the *hetF* mutant, UHM130, consistent with the idea that HetF is a CHF protease that requires these conserved active-site residues (Fig. 1D). Although pDR282 and pDR284 did not restore the ability of UHM130 to form heterocysts, some alleviation of the aberrant cell morphology was observed.

**Altered cell morphology of a *hetF* mutant requires *hetR* and *patA*.** The aberrant cell morphology of *hetF* deletion strain UHM130 is similar to that of the wild type in which *patA* is overexpressed ectopically (S. S. Young and S. M. Callahan unpublished data). Therefore, *hetF patA* and *hetF hetR* double-deletion mutants were constructed to determine if either *patA* or *hetR* contributes to the aberrant cell morphology of *hetF* deletion strain UHM130. Filaments of both double-mutant strains consisted of all vegetative cells with a wild-type morphology in both BG-11 and BG-11<sub>0</sub>, indicating that both *patA* and *hetR* are necessary for the aberrant cell morphology observed in the *hetF* mutant (data not shown).

**HetF and PatA affect HetR protein turnover.** Increased fluorescence from a *hetR-gfp* translational fusion in a *hetF* deletion strain suggested that mutation of *hetF* affects HetR protein levels in *N. punctiforme* (26). To characterize the effects of *hetF* and *patA* on HetR levels in *Anabaena* sp. strain PCC 7120,

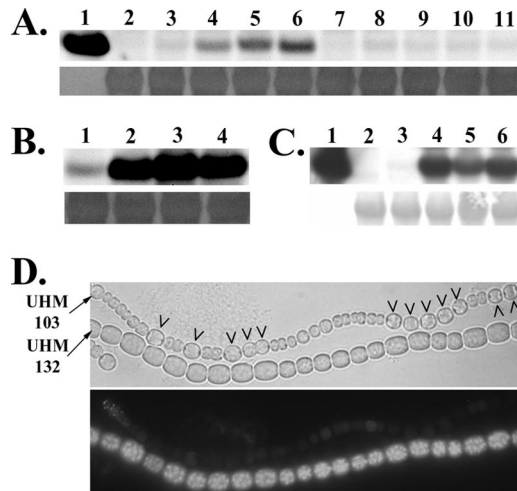


FIG. 2. Regulation of HetR levels by *patA* and *hetF*. Western blot analysis of HetR from cultures 18 h after the removal of combined nitrogen by a polyclonal antibody is shown in panel A. Shown are 100 ng of recombinant HetR used to generate the antibody (lane 1); UHM103, which is a  $\Delta$ *hetR* mutant (lane 2); *Anabaena* sp. strain PCC 7120, which is the wild type (lane 3); UHM101, which is a  $\Delta$ *patA* mutant (lane 4); UHM130, which is a  $\Delta$ *hetF* mutant (lane 5); UHM132, which is a  $\Delta$ *hetF*  $\Delta$ *patA* mutant (lane 6); UHM103 carrying *hetF* on replicating plasmid pDR267 (lane 7); *Anabaena* sp. strain PCC 7120 with pDR267 (lane 8); UHM101 with pDR267 (lane 9); UHM130 with pDR267 (lane 10); and UHM132 with pDR267 (lane 11). Western blot analysis of HetR expressed from the *petE* promoter on plasmid pPetHetR (5) 18 h after the removal of combined nitrogen and the addition of 3  $\mu$ M copper and detected by a polyclonal antibody is shown in panel B. Shown are UHM103, which is a  $\Delta$ *hetR* mutant with pPetHetR (lane 1); UHM109, which is a  $\Delta$ *hetR*  $\Delta$ *patA* mutant with pPetHetR (lane 2); UHM132, which is a  $\Delta$ *hetR*  $\Delta$ *hetF* mutant with pPetHetR (lane 3); and UHM134, which is a  $\Delta$ *hetR*  $\Delta$ *patA* mutant with pPetHetR (lane 4). Western blot analysis of HetRH6 expressed from the *nir* promoter on plasmid pDR204 18 h after the removal of 5 mM ammonium and addition of 17.6 mM nitrate with an antibody against the polyhistidine epitope tag is shown in panel C. Shown are 100 ng of recombinant HetRH6 (lane 1), UHM103 carrying untagged HetR on plasmid pDR216 (lane 2), UHM103 carrying epitope-tagged HetRH6 on plasmid pDR204 (lane 3), UHM109 carrying pDR204 (lane 4), UHM132 carrying plasmid pDR204 (lane 5), and UHM134 carrying plasmid pDR204 (lane 6). Chemiluminescence detection (upper panels) and a Coomassie-stained section of membrane corresponding to an approximate molecular mass of 50 kDa (lower panels) to show equality of protein loading for Western blot analyses are shown in panels A to C. Light (upper panel) and fluorescence (lower panel) microscopy of a mixture of filaments of UHM103, which is a  $\Delta$ *hetR* mutant, and UHM132, which is a  $\Delta$ *hetR*  $\Delta$ *hetF* mutant, both carrying pDR293, a plasmid bearing a *hetR-gfp* translational fusion expressed from  $P_{petE}$ , 24 h after the removal of combined nitrogen and the addition of 3  $\mu$ M copper is shown in panel D. Strains are indicated on the left. Carets indicate heterocysts.

a polyclonal antibody directed against HetR was used to assess protein levels in strains with or without each gene. Strains with one or both of the genes deleted from the chromosome had substantially higher levels of HetR than the wild type (Fig. 2A). The band for HetR from the wild type was very faint, whereas in the mutant backgrounds prominent bands were observed. In addition, the *hetF patA* double-deletion mutant had protein levels similar to those of the corresponding single mutants. Addition of a plasmid containing *hetF* to the mutant strains

restored HetR to a level similar to that of the wild-type strain (Fig. 2A).

To determine if the increase in HetR in cells lacking *hetF* or *patA* was due to either an effect on transcription of *hetR* or a posttranscriptional regulatory event, *hetR* was uncoupled from its normal promoter and fused to one of two inducible promoters. The fusions were then introduced on a plasmid into strains that, in addition to having either *hetF*, *patA*, or *hetF* and *patA* deleted, also had *hetR* deleted, so that the only source of HetR was from the gene fused to the inducible promoter. Plasmid-borne fusions of the *lacZ* and *gfp* reporter genes to the inducible promoters served as controls for potential plasmid or strain background effects on transcription levels, of which none were observed (data not shown). The inducible promoters used were the *petE* promoter, which is induced by copper in *Anabaena* sp. strain PCC 7120 (10), and the *nirA* promoter, which is induced by the presence of nitrate or lack of a fixed nitrogen source (9). When fused to *hetR* and introduced on a plasmid into either the wild type or a *hetR* deletion strain, both promoters cause the differentiation of supernumerary heterocysts in BG-11, which contains nitrate, or BG-11<sub>0</sub> under conditions that promote transcription (5, 21). The *nirA* promoter has also been used previously to evaluate levels of HetR made from various plasmid-borne mutant alleles of *hetR* (21). Whether expression of *hetR* was driven by the *petE* promoter in BG-11<sub>0</sub>, which contains copper, or the *nirA* promoter in BG-11, which contains nitrate, levels of HetR were dramatically increased in the genetic backgrounds that lacked *hetF*, *patA*, or both genes (Fig. 2B and C), indicating that deletion of *hetF* or *patA* affects the regulation of HetR levels posttranscription of *hetR*.

Wong and Meeks reported that wild-type *N. punctiforme* carrying a *hetR-gfp* translational fusion under the control of the *hetR* promoter displays fluorescence only in developing heterocysts, whereas the same construct in a *hetF* mutant has increased fluorescence distributed more uniformly throughout the filament. They suggest that HetF may be responsible for stabilizing HetR in heterocysts while promoting degradation in neighboring vegetative cells (26). However, the *hetR* promoter is known to be upregulated in developing heterocysts, complicating the interpretation of these results. To better characterize the spatial aspect of HetR turnover within filaments, a *hetR-gfp* translational fusion was expressed from the copper-inducible *petE* promoter in order to avoid the spatial pattern of transcription characteristic of the *hetR* promoter. A plasmid bearing a *P<sub>petE</sub>-hetR-gfp* translational fusion was introduced into a *hetR* deletion strain and a *hetR hetF* deletion strain. In BG-11<sub>0</sub>, which contains copper for the expression of *hetR-gfp* from the *petE* promoter, fluorescence was much brighter in the strain lacking *hetF*, consistent with the Western blotting results (Fig. 2D). In the strain with *hetF* intact, fluorescence was low in both heterocysts and vegetative cells, with only occasional vegetative cells displaying observable fluorescence (Fig. 2D). To control for potential differences in transcription from the *petE* promoter or GFP turnover in heterocysts and vegetative cells, a plasmid bearing a *P<sub>petE</sub>-gfp* transcriptional fusion, pSMC138, was introduced into both strains. Fluorescence in both strains was similar, and fluorescence resulting from the *P<sub>petE</sub>-hetR-gfp* fusion in the strain with *hetF* intact was lower in both vegetative cells and heterocysts relative to that from

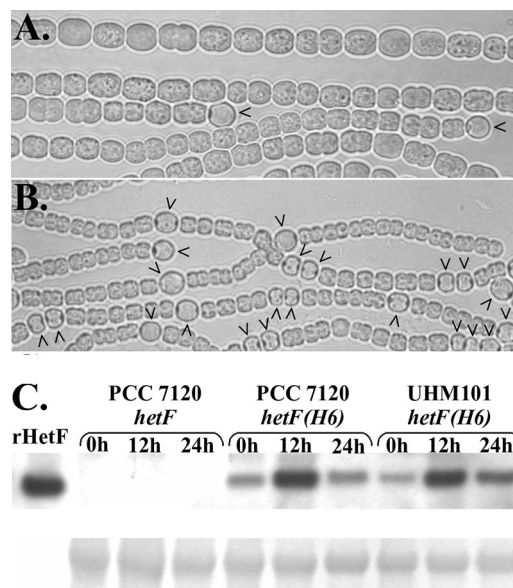


FIG. 3. Bypass of deletion of *patA* by extra copies of *hetF* and analysis of HetF protein levels. Shown is *patA* deletion strain UHM101 carrying control plasmid pAM505 (A) or pDR267, a plasmid bearing *P<sub>hetF</sub>-hetF* (B), 48 h after the removal of combined nitrogen. Carets indicate heterocysts. Also shown is a Western blot analysis of epitope-tagged HetF in *Anabaena* sp. strain PCC 7120 and *patA* deletion strain UHM101 at various times postinduction of differentiation (C). An antibody against the polyhistidine epitope tag was used to detect the protein. Lanes are as indicated. See the text for details. Chemiluminescent detection (upper panel) and a Coomassie-stained section of membrane (lower panel) were used to show equality of protein loading.

*Anabaena* sp. strain PCC 7120 carrying the *P<sub>petE</sub>-gfp* transcriptional fusion (data not shown), demonstrating that the low fluorescence in heterocysts generated by the *P<sub>petE</sub>-hetR-gfp* fusion was specifically due to HetR, not lower expression from the *petE* promoter or more rapid GFP turnover in developing heterocysts. Therefore, *hetF* limited the levels of HetR in both developing heterocysts and vegetative cells.

**Extra copies of *hetF* can functionally bypass deletion of *patA*.** A *patA* mutant strain forms heterocysts primarily at the filament termini (15), a phenotype that can be bypassed by deletion of *patS*, a negative regulator of differentiation (20), but not by extra copies or overexpression of *hetR*, the master regulator of differentiation (4, 5). To characterize the relationship between *hetF* and *patA*, pDR267, a replicating plasmid that contains *hetF* behind its native promoter, was introduced into a *patA* deletion strain, UHM101. UHM101 containing pDR267 formed intercalary heterocysts, and multiple contiguous heterocysts were occasionally observed within filaments when combined nitrogen was removed from the medium (Fig. 3A and B). As for the complemented *hetF* deletion strain, extra copies of *hetF* on a plasmid resulted in a decrease in the size of cells in filaments of the *patA* deletion strain.

Functional bypass of *patA* by extra copies of *hetF* could be explained by the regulation of HetF levels by PatA in the wild-type organism. To test if PatA affects HetF protein levels, the level of HetF in a wild-type genetic background was compared to that in one where *patA* had been deleted. Similar

amounts of HetF were detected by Western blot analysis of the wild-type strain, *Anabaena* sp. strain PCC 7120, and a *patA* deletion strain, UHM101, containing a plasmid with an epitope-tagged version of *hetF* under the control of its native promoter (Fig. 3C). Although no effect of deletion of *patA* on levels of HetF was seen, the level of HetF protein increased in both genetic backgrounds at 12 h after the induction of differentiation and returned to near preinduction levels by 24 h postinduction (Fig. 3C). In contrast, no significant differences in transcription from the *hetF* promoter region were observed at 0, 12, and 24 h after nitrogen step-down with promoter-*gfp* or promoter-*lacZ* fusions on plasmids pDR266 and pDR312, respectively (data not shown).

PatA has been proposed to attenuate *patS*- and *hetN*-dependent inhibitors of heterocyst formation based on the ability of strains with *patA* and *patS* or *patA*, *patS*, and *hetN* inactivated to form intercalary heterocysts (20). To test if HetF may have the same function, the *hetF* coding region was deleted from UHM100, a strain with *patS* deleted and *hetN* under the control of the copper-inducible *petE* promoter, to create strain UHM131. If the sole function of *hetF* is to attenuate one or both of these signals, then the resultant strain should be capable of forming heterocysts in BG-11<sub>0</sub> lacking copper, which prevents expression of *hetN* from the *petE* promoter. Strain UHM131 was unable to form heterocysts under these or any other conditions tested, suggesting that the function of *hetF* is not attenuation of either a *patS*- or a *hetN*-dependent signal (data not shown).

**HetF is required for differentiation exclusively in cells that differentiate.** Wong and Meeks have suggested that HetF is necessary for cell type-specific accumulation of HetR. Differential accumulation of HetR in cells along a filament by HetF could be the result of (i) limiting HetR levels in cells that will not differentiate, (ii) elevating levels of HetR in cells that will differentiate, or (iii) both i and ii. To test if the sole role of HetF in the differentiation of heterocysts is to limit levels of HetR in cells that will not differentiate, mosaic filaments were created that lacked *hetF* in all cells and had *hetR* in only one cell of the filament. If HetF is necessary for reduction of HetR only in cells adjacent to a differentiating cell, single cells that have *hetR* in a filament that otherwise lacks both *hetR* and *hetF* should be capable of differentiation, because the neighboring cells lack HetR, thereby obviating the need for HetF. A plasmid containing *hetR* expressed from the *nirA* promoter and a transcriptional fusion between the *petE* promoter and *gfp* was introduced into a *hetF* *hetR* double mutant under conditions that allowed the examination of filaments in which only one cell of the filaments had received the plasmid (see Materials and Methods). Fluorescence generated as a result of the P<sub>*petE*</sub>-*gfp* fusion was used to identify cells that had received the plasmid. More than 100 filaments with a single fluorescent cell were examined, and none of the fluorescent cells had differentiated into a heterocyst (Fig. 4A). Many of these cells were, however, enlarged, consistent with the phenotype of a *hetF* mutant strain with an active copy of *hetR*. The same plasmid introduced into a *hetR* deletion strain resulted in differentiation of almost all of the cells that were the sole fluorescent cells of a filament (Fig. 4B).

To test if *hetF* is required exclusively in cells that will differentiate into heterocysts, mosaic filaments in which only one cell

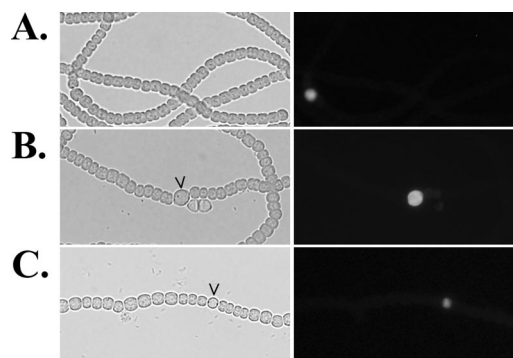


FIG. 4. GFP-tagged plasmids bearing *hetR* or *hetF* in one cell of a filament. Shown is light (left panel) and fluorescence (right panel) microscopy of UHM132, a *hetR hetF* deletion strain with *hetR* and *gfp* on plasmid pDR309 in a single cell of a filament in BG-11 medium (A); UHM103, a *hetR* deletion strain with plasmid pDR309 in a single cell of a filament in BG-11 medium (B); and UHM130, a *hetF* deletion strain with *hetF* and *gfp* on plasmid pDR310 in a single cell of a filament in BG11<sub>0</sub> medium (C). GFP fluorescence identifies cells that have the indicated plasmid. Carets indicate heterocysts.

contained an active copy of *hetF* were constructed. A plasmid containing *hetF* under the control of its native promoter and a P<sub>*petE*</sub>-*gfp* fusion was introduced into a *hetF* deletion strain under conditions that allowed the examination of filaments in which only one cell of the filaments had received the plasmid. In this case, approximately 25% of the 100 cells examined that were the sole fluorescent cells of a filament had differentiated into heterocysts (Fig. 4C). Within these filaments, cells adjacent to the heterocysts often lacked the aberrant cell morphology typical of this strain, although no distinguishable fluorescence could be observed in the adjacent cells, suggesting that a diffusible substance such as that proposed to be produced by *patS* may be responsible for this phenomenon. This hypothesis is supported by the fact that when added to the medium, the C-terminal pentapeptide PatS-5 can alleviate the aberrant cell morphology of *hetF* deletion strain UHM130 (data not shown) and that induction of transcription of *patS* is dependent on *hetF* (see below). HetF was required for differentiation exclusively in developing heterocysts, and its control of HetR levels in vegetative cells adjacent to a developing cell was not necessary for differentiation.

**Transcription from the *patS* promoter and the -271 TSP of the *hetR* promoter is dependent on *hetF*.** Transcription from the *hetR* promoter relies on HetR both directly and indirectly. HetR is required for the use of, and binds just upstream of, the -271 TSP of the *hetR* promoter (13, 18). In addition, HetR is part of an indirect positive feedback loop with NtcA and NrrA, which are necessary for the use of other TSPs in the *hetR* promoter (7, 18). Therefore, an increase in the levels of HetR in a *hetF* mutant might be expected to increase the transcription of *hetR*, yet Wong and Meeks showed with a Northern blot assay that transcription of *hetR* is reduced in a *hetF* mutant (26). To test if *hetF* is required for the regulation of transcription of *hetR*, a highly sensitive RACE protocol was used to detect transcription from all four TSPs of the *hetR* promoter in *Anabaena* sp. strain PCC 7120; a *hetF* deletion strain; a strain carrying *hetR*(S179N) in the chromosome, which is incapable of promoting transcription from the -271 TSP; and a strain

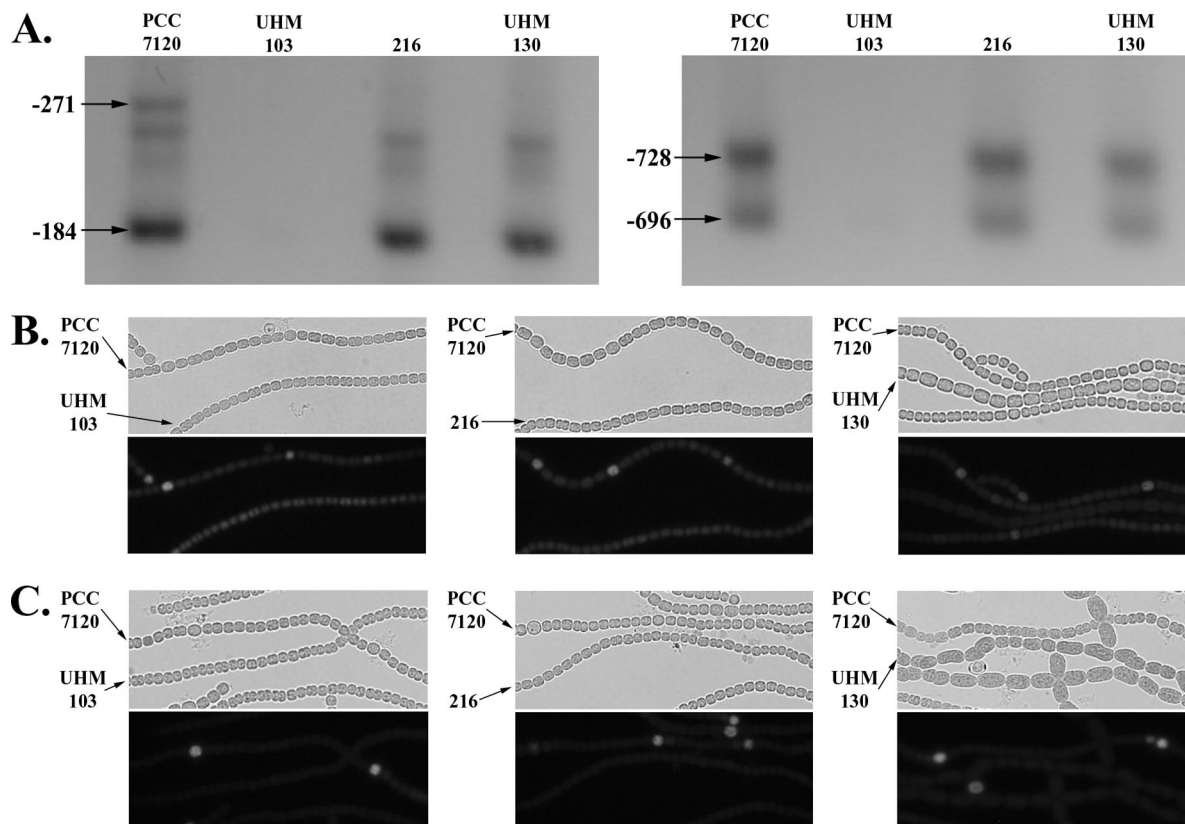


FIG. 5. Effect of mutation of *hetF* and *hetR* on transcription from the  $-271$  TSP of *hetR* and transcription from the *hetR* and *patS* promoters. Agarose gel electrophoresis of RACE products from the  $-271$  and  $-184$  (left panel) and  $-728$  and  $-696$  (right panel) TSPs is shown in panel A; lanes are as indicated. Also shown is light (upper panel) and fluorescence (lower panel) microscopy of a mixture of filaments of *Anabaena* sp. strains PCC 7120 and UHM103, which is a  $\Delta$ *hetR* mutant; strain 216, which carries the *hetR*(*S179R*) allele, or UHM130, which is a  $\Delta$ *hetF* mutant carrying pSMC127, a plasmid bearing  $P_{hetR}$ -*gfp* (B), or pAM1951, a plasmid bearing  $P_{patS}$ -*gfp* (C). Strain designations are indicated by arrows.

with both the *hetR* promoter and coding regions deleted. Transcription from the  $-728$ ,  $-696$ , and  $-184$  start sites was detected from all strains but the *hetR* deletion strain, indicating that *hetF* was not required for their use (Fig. 5A). On the other hand, transcripts for the  $-271$  start site were present only in *Anabaena* sp. strain PCC 7120, indicating that *hetF* was required for *hetR*-dependent positive autoregulation of the  $-271$  TSP (Fig. 5A). A third PCR product slightly smaller than that of the  $-271$  TSP was observed for some strains. However, when sequenced, this product corresponded to the  $-184$  TSP, suggesting that nonspecific priming, not another TSP, was responsible for this product.

Transcription of *patS*, as well as *hetR*, is dependent on *hetR*. To test the effect of deletion of *hetF* on patterned induction of *patS* and *hetR* transcription in vivo, patterns of fluorescence from transcriptional fusions between *gfp* and the two promoters were examined in different strains carrying plasmids pAM1951 and pSMC127, respectively. Patterned expression from a  $P_{hetR}$ -*gfp* fusion after nitrogen step-down was observed only in *Anabaena* sp. strain PCC 7120. Uniform fluorescence at a level similar to that of the vegetative cells of *Anabaena* sp. strain PCC 7120 was observed in all cells of the *hetR* deletion, *hetR*(*S179N*), and *hetF* deletion strains (Fig. 5B). Similarly, patterned expression from a  $P_{patS}$ -*gfp* fusion after nitrogen step-down was observed only in *Anabaena* sp. strain PCC 7120,

with little fluorescence detected in the other three strains (Fig. 5C). A negative feedback loop between *hetR* and an inhibitor, such as that produced by *patS* or *hetN*, could have accounted for the decrease in *hetR* and *patS* expression seen in the *hetF* deletion and *hetR*(*S179N*) strains. However, when both fusions were introduced into a *hetF patS* double-deletion strain with transcription of *hetN* controlled by a copper-inducible promoter and the strain was examined in BG-11<sub>0</sub> lacking copper, no increase in expression from the *hetR* or *patS* promoter was observed, suggesting that negative feedback between *hetR* and *patS* or *hetN* was not responsible for the lack of induction of the *hetR* and *patS* promoters in a *hetF* deletion strain (data not shown).

## DISCUSSION

Since the report of its discovery, HetF has been proposed to be a protease of the CHF class, specifically, the separinoids (1). Our results are consistent with this prediction. Conserved active-site histidine and cysteine residues of CHF proteases were essential for HetF function, and deletion of *hetF* caused a dramatic increase in the level of HetR. In addition to HetF, deletion of *patA* caused HetR to accumulate to a similar level. The available evidence suggests that the two proteins work together to regulate levels of HetR, with PatA facilitating the



activity of HetF, which, unlike that of PatA, was absolutely required for cellular differentiation. Simultaneous deletion of both *patA* and *hetF* did not lead to a further increase in HetR protein, and the addition of *hetF* to a *patA* deletion strain functionally bypassed *patA* to restore intercalary heterocysts and decrease the level of HetR. The role of PatA in this pathway is unclear. PatA was not necessary for wild-type levels of HetF, suggesting that it may upregulate HetF activity, possibly through mediating protein-protein interactions, given that this is the predicted function of the N-terminal PATAN domain found in PatA (16). Alleles of *hetR* bearing S179N, G36A, and H69Y substitutions have been shown to result in increased HetR protein levels (21, 32), and the level of HetR observed under each of these conditions is similar to that seen after the deletion of *hetF* or *patA* in a wild-type *hetR* background (D. D. Risser and S. M. Callahan, unpublished data), suggesting that they may affect the same HetF-dependent pathway. Unlike HetR accumulation with the mutant forms of HetR, which are nonfunctional, deletion of *hetF* or *patA* causes a dramatic increase in the level of wild-type HetR. Therefore, HetF and PatA are required for differentiation even when a large excess of wild-type HetR protein is present.

The role of HetR degradation in heterocyst formation has been somewhat enigmatic. HetR is reported to be a  $\text{Ca}^{2+}$ -dependent serine protease capable of autodegradation with the active-site serine located at residue 152 and an additional serine at residue 179 also required for protease activity (33). However, the proposed active-site serine is not required for proper HetR turnover or heterocyst formation (21). Also, in vivo, depletion of calcium does not result in an increase in HetR protein similar to that observed for a strain carrying *hetR*(S179N) in the chromosome, strain 216, even though autodegradation of HetR should be abolished in both backgrounds if calcium is necessary for autodegradation of HetR (31, 32). These inconsistencies cast doubt on the assertion that the serine at residue 179 of HetR is required for protease activity and make it difficult to determine what effect, if any, HetR protease activity may have on *hetF*-dependent turnover of HetR.

Wong and Meeks speculated that the mechanism by which HetF promotes differentiation may involve cell-specific accumulation of HetR via regulation of HetR autoproteolytic activity (26). Evidence suggesting that HetF is itself a protease suggests that it may have a more direct role in the degradation of HetR. HetF was necessary for limiting HetR levels in both vegetative cells and developing heterocysts, and *hetF* appears to be expressed at similar levels in the two cell types. The multicellular nature of *Anabaena* sp. strain PCC 7120 suggests that *hetF* activity could be required in either or both cell types for differentiation of cells. For instance, HetF may be necessary for decreasing HetR levels in some cells so that others can differentiate. Induction of expression of *patS* is dependent on *hetR* (13), and overexpression of both genes prevents the differentiation of any cells in a filament (20). Alternatively, HetF may convert a HetR apoprotein to its active form to promote the differentiation of a cell. Determining which role HetF might play is nontrivial and difficult to do with current methodologies. For example, simply using cell type-specific promoters to drive the expression of *hetF* and checking for complementation of a mutant will not work because expression from

such promoters is dependent on the differentiation process, which is stopped at an early stage in a *hetF* mutant. Instead, mosaic filaments were examined in which *hetF* was expressed in only one cell of a filament to show that it can promote the differentiation of that cell even when neighboring cells lack HetF activity. In addition, it was shown that in a *hetF* deletion background, HetR was not sufficient to promote the differentiation of a cell when the neighboring cells lacked HetR. Taken together, these results indicate that HetF acts to promote the differentiation of heterocysts along a filament in cells that will differentiate. These results do not preclude a possible role for HetF in vegetative cells in patterning.

HetF from *Anabaena* sp. strain PCC 7120 appears to serve a function similar to that of HetF from *N. punctiforme* and is required for heterocyst formation. Unlike *N. punctiforme*, however, deletion of *hetF* from *Anabaena* sp. strain PCC 7120 results in aberrant cell morphology as well. It should be noted that when isolating the *hetF* deletion strain, only three of four isolates tested displayed this aberrant cell morphology, even though all four could be complemented by reintroduction of *hetF*. Furthermore, a strain isolated from an independent study displayed aberrant cell morphology identical to that of *hetF* deletion strain UHM130 and was found to have a transposon inserted within the *hetF* coding region (Risser and Callahan, unpublished). When working with UHM130, presumptive mutants with this phenotype suppressed were also isolated frequently, suggesting a selective pressure to acquire suppressor mutations that abrogate the aberrant cell morphology. Isolation of a suppressor mutant may explain why this phenotype was not observed when *hetF* was inactivated in *N. punctiforme*. Transcriptional expression of *hetF* is constitutive and unpatterned in both *N. punctiforme* and *Anabaena* sp. strain PCC 7120, but in *Anabaena* sp. strain PCC 7120, at least, the level of HetF protein increases transiently after the removal of combined nitrogen, suggesting some temporal, and perhaps spatial, regulation of HetF in response to nitrogen starvation. Unfortunately, an initial attempt to observe HetF in vivo with a *hetF-gfp* translational fusion was unsuccessful due to a lack of observable fluorescence, although this fusion was capable of complementing *hetF* deletion strain UHM130 (Risser and Callahan, unpublished). It is unclear if the lack of detectable fluorescence is due to improper folding of GFP or if the level of HetF is simply too low to be observed by such methods.

HetF is necessary for the proper regulation of HetR levels at both the transcriptional and posttranscriptional levels. Transcription from the  $-271$  start point of the *hetR* promoter and transcription from the *patS* promoter are dependent on *hetR*, and HetR has been shown to bind to regions within each of these promoters (13, 18). In *hetF* deletion strain UHM130, although there is an abundance of HetR protein, no transcription from these sites is observed. This is somewhat surprising given that *hetR* acts stoichiometrically; overexpression of *hetR* in a wild-type strain results in an increase in heterocyst formation. This inverse correlation between the effects of HetF on the level of HetR protein and transcription from *hetR*-dependent promoters suggests two possibilities: (i) that a *hetF*-dependent product acts with HetR to promote transcription from *hetR*-dependent promoters, after which HetR is concomitantly degraded, and (ii) that *hetF* acts directly or indirectly to convert a HetR apoprotein to an active form that can then pro-

mote transcription from *hetR*-dependent promoters. The latter possibility is particularly appealing considering evidence suggesting that HetF is a protease.

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#### REFERENCES

- Aravind, L., and E. V. Koonin. 2002. Classification of the caspase-hemoglobin fold: detection of new families and implications for the origin of the eukaryotic separins. *Proteins* **46**:355–367.
- Black, T. A., Y. Cai, and C. P. Wolk. 1993. Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. *Mol. Microbiol.* **9**:77–84.
- Borthakur, P. B., C. C. Orozco, S. S. Young-Robbins, R. Haselkorn, and S. M. Callahan. 2005. Inactivation of *patS* and *hetN* causes lethal levels of heterocyst differentiation in the filamentous cyanobacterium *Anabaena* sp. PCC 7120. *Mol. Microbiol.* **57**:111–123.
- Buikema, W. J., and R. Haselkorn. 1991. Characterization of a gene controlling heterocyst development in the cyanobacterium *Anabaena* 7120. *Genes Dev.* **5**:321–330.
- Buikema, W. J., and R. Haselkorn. 2001. Expression of the *Anabaena* *hetR* gene from a copper-regulated promoter leads to heterocyst differentiation under repressing conditions. *Proc. Natl. Acad. Sci. USA* **98**:2729–2734.
- Callahan, S. M., and W. J. Buikema. 2001. The role of HetN in maintenance of the heterocyst pattern in *Anabaena* sp. PCC 7120. *Mol. Microbiol.* **40**:941–950.
- Ehira, S., and M. Ohmori. 2006. NrrA directly regulates expression of *hetR* during heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* **188**:8520–8525.
- Elhai, J., and C. P. Wolk. 1988. Conjugal transfer of DNA to cyanobacteria. *Methods Enzymol.* **167**:747–754.
- Frias, J. E., E. Flores, and A. Herrero. 1997. Nitrate assimilation gene cluster from the Heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* **179**:477–486.
- Ghassemian, M., B. Wong, F. Ferreira, J. L. Markley, and N. A. Straus. 1994. Cloning, sequencing and transcriptional studies of the genes for cytochrome *c*-553 and plastocyanin from *Anabaena* sp. PCC 7120. *Microbiology* **140**:1151–1159.
- Golden, J. W., and H.-S. Yoon. 2003. Heterocyst development in *Anabaena*. *Curr. Opin. Microbiol.* **6**:557–563.
- Higuchi, R., B. Krummel, and R. K. Saiki. 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* **16**:7351–7367.
- Huang, X., Y. Dong, and J. Zhao. 2004. HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS. *Proc. Natl. Acad. Sci. USA* **101**:4848–4853.
- Khudyakov, I. Y., and J. W. Golden. 2004. Different functions of HetR, a master regulator of heterocyst differentiation in *Anabaena* sp. PCC 7120, can be separated by mutation. *Proc. Natl. Acad. Sci. USA* **101**:16040–16045.
- Liang, J., L. Scappino, and R. Haselkorn. 1992. The *patA* gene product, which contains a region similar to CheY of *Escherichia coli*, controls heterocyst pattern formation in the cyanobacterium *Anabaena* 7120. *Proc. Natl. Acad. Sci. USA* **89**:5655–5659.
- Makarova, K. S., E. V. Koonin, R. Haselkorn, and M. Y. Galperin. 2006. Cyanobacterial response regulator PatA contains a conserved N-terminal domain (PATAN) with an alpha-helical insertion. *Bioinformatics* **22**:1297–1301.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Muro-Pastor, A. M., A. Valladares, E. Flores, and A. Herrero. 2002. Mutual dependence of the expression of the cell differentiation regulatory protein HetR and the global nitrogen regulator NtcA during heterocyst development. *Mol. Microbiol.* **44**:1377–1385.
- Nayar, A. S., H. Yamaura, R. Rajagopalan, D. D. Risser, and S. M. Callahan. 2007. FraG is necessary for filament integrity and heterocyst maturation in the cyanobacterium *Anabaena* sp. strain PCC 7120. *Microbiology* **153**:601–607.
- Orozco, C. C., D. D. Risser, and S. M. Callahan. 2006. Epistasis analysis of four genes from *Anabaena* sp. strain PCC 7120 suggests a connection between PatA and PatS in heterocyst pattern formation. *J. Bacteriol.* **188**:1808–1816.
- Risser, D. D., and S. M. Callahan. 2007. Mutagenesis of *hetR* reveals amino acids necessary for HetR function in the heterocystous cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* **189**:2460–2467.
- Schaefer, M. R., and S. S. Golden. 1989. Differential expression of members of a cyanobacterial *psbA* gene family in response to light. *J. Bacteriol.* **171**:3973–3981.
- Wei, T.-F., R. Ramasubramanian, and J. W. Golden. 1994. *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. *J. Bacteriol.* **176**:4473–4482.
- Wolk, C. P., A. Ernst, and J. Elhai. 1994. Heterocyst metabolism and development, p. 769–823. In D. A. Bryant (ed.), *The molecular biology of cyanobacteria*, vol. 1. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Wolk, C. P., Q. Fan, R. Zhou, G. Huang, S. Lechno-Yossef, T. Kuritz, and E. Wojciuch. 2007. Paired cloning vectors for complementation of mutations in the cyanobacterium *Anabaena* sp. strain PCC 7120. *Arch. Microbiol.* **188**:551–563.
- Wong, F. C. Y., and J. C. Meeks. 2001. The *hetF* gene product is essential to heterocyst differentiation and affects HetR function in the cyanobacterium *Nostoc punctiforme*. *J. Bacteriol.* **183**:2654–2661.
- Wu, X., D. Liu, M. H. Lee, and J. W. Golden. 2004. *patS* minigenes inhibit heterocyst development of *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* **186**:6422–6429.
- Yoon, H.-S., and J. W. Golden. 1998. Heterocyst pattern formation controlled by a diffusible peptide. *Science* **282**:935–938.
- Yoon, H.-S., and J. W. Golden. 2001. PatS and products of nitrogen fixation control heterocyst pattern. *J. Bacteriol.* **183**:2605–2613.
- Zhang, C.-C., S. Laurent, S. Sakr, L. Peng, and S. Bedu. 2006. Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. *Mol. Microbiol.* **59**:367–375.
- Zhao, Y., Y. Shi, W. Zhao, X. Huang, D. Wang, N. Brown, J. Brand, and J. Zhao. 2005. CcbP, a calcium-binding protein from *Anabaena* sp. PCC 7120, provides evidence that calcium ions regulate heterocyst differentiation. *Proc. Natl. Acad. Sci. USA* **102**:5744–5748.
- Zhou, R., Z. Cao, and J. Zhao. 1998. Characterization of HetR protein turnover in *Anabaena* sp. PCC 7120. *Arch. Microbiol.* **169**:417–423.
- Zhou, R., X. Wei, N. Jiang, H. Li, Y. Dong, K.-L. Hsi, and J. Zhao. 1998. Evidence that HetR is an unusual serine-type protease. *Proc. Natl. Acad. Sci. USA* **95**:4959–4963.