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Expanding the Direct HetR Regulon in *Anabaena* sp. Strain PCC 7120

Patrick Videau,[a] Shuisong Ni,[b] Orion S. Rivers,[a] Blake Ushijima,[a] Erik A. Feldmann,[c] Loralyn M. Cozy,[a] Michael A. Kennedy,[b] Sean M. Callahan[a]

Department of Microbiology, University of Hawaii, Honolulu, Hawaii, USA[a]; Department of Chemistry and Biochemistry, Miami University, Oxford, Ohio, USA[b]; Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri, USA[c]

In response to a lack of environmental combined nitrogen, the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 differentiates nitrogen-fixing heterocyst cells in a periodic pattern. HetR is a transcription factor that coordinates the regulation of this developmental program. An inverted repeat-containing sequence in the *hepA* promoter required for proheterocyst-specific transcription was identified based on sequence similarity to a previously characterized binding site for HetR in the promoter of *hetP*. The binding affinity of HetR for the *hepA* site is roughly an order of magnitude lower than that for the *hetP* binding site. A BLAST search of the *Anabaena* genome identified 166 *hepA*-like sites that occur as single or tandem sites (two binding sites separated by 13 bp). The vast majority of these sites are present in predicted intergenic regions. HetR bound five representative single binding sites *in vitro*, and binding was abrogated by transversions in the binding sites that conserved the inverted repeat nature of the sites. Binding to four representative tandem sites was not observed. Transcriptional fusions of the green fluorescent protein gene *gfp* with putative promoter regions associated with the representative binding sites indicated that HetR could function as either an activator or repressor and that activation was cell-type specific. Taken together, we have expanded the direct HetR regulon and propose a model in which three categories of HetR binding sites, based on binding affinity and nucleotide sequence, contribute to three of the four phases of differentiation.

In 1961, Monod and Jacob postulated that differentiation was the sustained change in gene expression leading to a change in morphology (1). This definition has held true for many developmental programs, including endospore formation in *Bacillus* and *Myxococcus* species, aerial mycelium and spore formation in *Streptomyces* species, and the formation of stalk cells in *Caulobacter* species. To mediate the changes in gene expression necessary for the production of these specialized structures, each of these model organisms relies on a global or master regulator of differentiation (Spo0A in *Bacillus subtilis* [2, 3], FruA in *Myxococcus xanthus* [4, 5], AdpA in *Streptomyces griseus* [6, 7], and CtrA in *Caulobacter crescentus* [8, 9]). These regulators directly interact with the promoters of a few to many hundred genes, termed regulons, to either activate or repress transcription, coordinating the process of differentiation. While much work has focused on describing the regulons of the aforementioned regulators, comparatively little is known about the regulon of the master regulator of heterocyst differentiation in *Anabaena* sp. strain PCC 7120 (hereinafter called *Anabaena*).

Anabaena is a filamentous cyanobacterium that responds to low levels of combined nitrogen by differentiating specialized heterocyst cells that provide a microoxic environment for the fixation of dinitrogen by the oxygen-labile nitrogenase complex (reviewed in references 10, 11, and 52). Heterocysts are morphologically distinct cells that develop at semiregular intervals and are separated by approximately 10 to 20 photosynthetic vegetative cells, resulting in a 1-dimensional pattern along filaments. This differentiation process results in a change in the transcription of roughly 1,500 genes, which is facilitated by HetR, the master regulator of differentiation (12). A *hetR* deletion in *Anabaena* results in the inability to develop heterocysts, whereas overexpression yields supernumerary heterocysts even under nitrogen-replete conditions (13, 14).

HetR acts as a transcriptional regulator that functions early in the regulatory cascade governing differentiation. Recent work mapping all of the transcriptional start sites (TSSs) in *Anabaena* has identified 209 TSSs that are differentially regulated in wild-type and *hetR* mutant strains; expression from these TSSs was >8-fold higher in the wild type than in a *hetR* mutant strain (15). The regulation by HetR of many of these TSSs is likely indirect. HetR has been shown to bind to large DNA fragments (>150 bp) from the promoters of *patS*, *hepA*, *hetR* (16), and *pknE* (17), as well as to 29-bp and 40-bp DNA fragments derived from the promoters of *hetP* (18) and *hetZ* (19), respectively, *in vitro*, suggesting direct regulation of the transcription of these genes.

The ability of HetR to bind DNA is likely required for its regulatory function. The initial structure of the *Fischerella* HetR dimer, shown to complement an *Anabaena hetR* mutant, has been solved and displays four domains (20). Two flap domains extend outwards from the sides of the structure and are thought to mediate protein-protein interactions. The hood domain, which includes both C termini, likely interacts with a diffusible peptide (21, 22) that is derived from two inhibitors of differentiation (PatS and HetN) and promotes the degradation of HetR (23). The N termini create a DNA-binding domain containing helix-turn-helix motifs. Recently, cocrystallization of *Fischerella* HetR bound to a 21-bp DNA fragment based on that from the *hetP* promoter has identified the necessary protein-DNA interactions that confer DNA binding specificity to HetR (12). Most strikingly, the interaction of Glu71 with three consecutive cytosines during DNA

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Address correspondence to Sean M. Callahan, scallaha@hawaii.edu.
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binding defines the requirement of an inverted repeat-containing sequence with CCC-N_5-GGG at its core. An allele of HetR with Glu71 mutated was unable to bind DNA *in vitro* or complement an *Anabaena hetR* mutant strain, illustrating the absolute requirement of this amino acid for proper HetR function.

Clearly defining the HetR regulon would provide insight not only into the exact function of HetR but also into the cascade of events driving cellular differentiation. Here, we report the identification of a 17-bp inverted repeat-containing sequence in the *hepA* promoter that was bound by HetR *in vitro* and necessary for transcription *in vivo*. Additional HetR binding sites were uncovered by similarity to the *hepA* site, representatives of which were bound by HetR and used in transcriptional fusions to show that HetR can act as either an activator or repressor. These results suggest complex regulation of the HetR regulon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The growth of *Escherichia coli* and *Anabaena* sp. strain PCC 7120 (wild type) and its derivatives, the concentrations of antibiotics, the induction of heterocysts in medium lacking a source of combined nitrogen, and the conditions for photomicroscopy were as previously described (18, 24). Plasmids were introduced into *Anabaena* strains by conjugation from *E. coli* as previously described (25).

Plasmid and strain construction. The strains and plasmids used in this study are listed in Table 1. The primers used in this study are listed in Table S1 in the supplemental material. The integrity of all PCR-derived constructs was verified by sequencing. The transcriptional promoter fusions to the green fluorescent protein gene (*gfp*) were designed to include at least the nearest TSS upstream from the chosen genes as defined by Mitschke et al. (15). Plasmid and strain construction is detailed in the supplemental material.

Purification of recombinant HetR and DNA binding assays. Recombinant HetR was overexpressed and purified from BL21(DE3) cells as previously described (22). Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously except that SYBR green, instead of ethidium bromide, was utilized according to the manufacturer's instructions for imaging gels to estimate K_d (dissociation constant) (22). The preparation of the 29-bp DNA fragments used for binding experiments is detailed in the supplemental material.

Fluorescence quantification. Green and red fluorescence from *Anabaena* was quantified with a GloMax-Multi Jr detection system (Promega) using the blue and red fluorometer kits, respectively, according to the manufacturer's instructions. Initially, standard curves of the optical density at 750 nm (OD_{750}) versus both green and red autofluorescence were generated for each tested *Anabaena* strain containing pAM1956 (26). The optimal OD_{750} for measurement of both green and red fluorescence was found to be 0.1 for all strains, with an acceptable range of 0.05 to 0.15; beyond 0.15, the correlation between autofluorescence and OD_{750} was nonlinear. For *gfp* fluorescence measurements, 20-ml liquid cultures of each tested strain containing a transcriptional fusion to *gfp* were grown in triplicate to an OD_{750} of 0.5 to 0.8. Aliquots from each culture were diluted to an OD_{750} of 0.1 in either BG-11 or BG-11_0 (BG-11 without a source of fixed nitrogen) to a final volume of 1 ml, and red and green fluorescence was measured. Red autofluorescence measurements were used to validate the OD_{750} of 0.1. Using the standard curves, green background fluorescence was subtracted from the tested strains containing a transcriptional fusion to *gfp* to yield a measurement of green fluorescence in fluorescence standard units (FSU).

Phylogenetic analysis. Individual groups of the single and tandem *hepA*-like binding sites, generally 29 bp and 59 bp, respectively, were aligned with ClustalW. A maximum likelihood tree was constructed for each group using the generalized time-reversible algorithm (27) and 1,000 bootstrap replicates were performed using MEGA5 (28).

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Anabaena strains		
PCC 7120	Wild type	Pasteur Culture Collection
UHM101	Δ*patA*	36
UHM103	Δ*hetR*	24
UHM244	Δ*hetR*(E71N)	12
UHM269	Δ*asr0081*::ΩSp^r/Sm^r	This study
UHM270	Δ*all1321*::ΩSp^r/Sm^r	This study
UHM273	Δ*alr1000*::ΩSp^r/Sm^r	This study
UHM274	Δ*all1748*::ΩSp^r/Sm^r	This study
UHM275	Δ*alr1347*::ΩSp^r/Sm^r	This study
UHM276	Δ*all5218*::ΩSp^r/Sm^r	This study
UHM277	Δ*asl0206*::ΩSp^r/Sm^r	This study
UHM278	Δ*all3746*::ΩSp^r/Sm^r	This study
Plasmids		
pAM504	Shuttle vector for replication in *E. coli* and *Anabaena*; Km^r Nm^r	53
pAM1956	Shuttle vector pAM504 with promoterless *gfp*	26
pRL278	Suicide vector; Km^r Nm^r	49
pDW9	Source of Sp^r/Sm^r Ω interposon	54
pET28b+*hetR*	Expression vector for purifying polyhistidine epitope-tagged HetR; Km^r	22
pPJAV127	pAM504 with P_{hepA} transcriptionally fused to *gfp*	This study
pPJAV128	pAM504 with $P_{all0961}$ transcriptionally fused to *gfp*	This study
pPJAV132	pAM504 with $P_{alr1347}$ transcriptionally fused to *gfp*	This study
pPJAV210	pAM504 with P_{hepA} with a mutated HetR binding site	This study
pPJAV203	pAM1956 with $P_{asr0081}$	This study
pPJAV204	pAM1956 with $P_{asl0206}$	This study
pPJAV205	pAM1956 with $P_{alr1000}$	This study
pPJAV206	pAM1956 with $P_{all1321}$	This study
pPJAV207	pAM1956 with $P_{all1748}$	This study
pPJAV208	pAM1956 with $P_{all3746}$	This study
pPJAV209	pAM1956 with $P_{all5218}$	This study
pPJAV210	pAM1956 with $P_{rrn16Sa}$	This study
pPJAV162	pRL278 used to make UHM277	This study
pPJAV163	pRL278 used to make UHM278	This study
pPJAV164	pRL278 used to make UHM275	This study
pPJAV165	pRL278 used to make UHM273	This study
pPJAV166	pRL278 used to make UHM274	This study
pPJAV167	pRL278 used to make UHM270	This study
pPJAV168	pRL278 used to make UHM276	This study
pPJAV200	pRL278 used to make UHM269	This study
pPJAV201	Suicide plasmid used to replace *all0961* with an Sp^r/Sm^r interposon insertion	This study

[a] ΩSp^r/Sm^r, interposon conferring resistance to spectinomycin and streptomycin. Nm, neomycin.

Acetylene reduction assays, thin-layer chromatography, and alcian blue staining. Aerobic and anaerobic acetylene reduction assays were performed as previously described (24, 29). The positive-control Δ*pbp6* strain used in the anaerobic assays was a transposon mutant that will be published elsewhere (R. Oshiro, L. M. Cozy, and S. M. Callahan, unpublished data). Lipid extractions were performed 24 h after the induction of

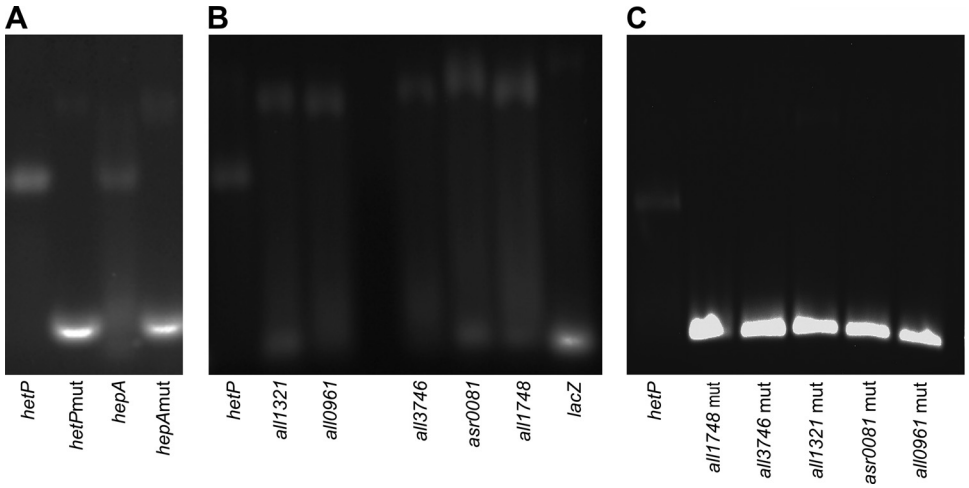

FIG 1 (A, B) HetR binds to 29-bp DNA fragments containing *hepA* (A) and representative single (B) binding sites in electrophoretic mobility shift assays. (C) HetR no longer bound single binding sites containing transversion mutations in each of the 17-bp palindromes. In all lanes, 10 μM HetR and 5 μM DNA were used. The gene name below each lane denotes the promoter region from which the respective 29-bp DNA fragment was derived.

heterocyst differentiation and run on thin-layer chromatography plates as previously described (30). The plates were developed at 120°C for 1 h. Heterocyst-specific exopolysaccharide was stained with alcian blue as previously described (18).

RESULTS

HetR binds to an inverted repeat in the *hepA* promoter required for normal transcription. To date, a defined nucleotide sequence required for HetR binding has only been shown for two sites (18, 19). Both sites contain the binding motif CCC-N_5-GGG and are located in the promoter regions of the *hetP* and *hetZ* genes. To identify additional HetR binding sites in the *Anabaena* genome, the promoter region DNA sequences of genes whose expression is regulated by HetR were searched for the binding motif CCC-N_5-GGG *in silico*. Within the promoter regions of the *patA*, *patS*, *hetR*, *ntcA*, *hetP*, *hetZ*, *trpE*, and *hepA* genes, 14 occurrences of this motif were found. To test for DNA-protein interaction, an electrophoretic mobility shift assay was conducted with HetR and 29-bp DNA fragments containing the 14 CCC-N_5-GGG motifs. A shifted species, indicative of HetR binding to the DNA, was present for 29-bp fragments derived from the *hetZ*, *trpE*, and *hepA* promoter regions (Fig. 1A and data not shown). Binding of HetR to the *hetZ* binding site has previously been shown (19), and the results from the *trpE* binding site will be published elsewhere. The third fragment bound by HetR contained a 17-bp inverted repeat sequence, herein referred to as the binding site, centered 511 bases upstream from the *hepA* coding region. The similarly shifted species in the *hetP* and *hepA* lanes show that HetR binds to the *hepA* binding site *in vitro* (Fig. 1A).

The sequence specificity of HetR for the *hetP* binding site was assessed by Higa and Callahan (18) by mutation of six nucleotides within the binding site that abrogated specific binding and were later found to be primary sites of interaction between HetR and this DNA (12). To test the sequence specificity of the *hepA* binding site, the binding of HetR to a 29-bp DNA fragment with transversions at each of the nucleotides comprising the 17-bp *hepA* binding site core, which maintained the inverted repeat structure, was visualized by EMSA (Fig. 1A; see also Fig. S1 in the supplemental material). Similar to the results for mutated *hetP* binding site that remained unbound by HetR, no shift was seen with the mutated *hepA* binding site. While faint bands are present in the lanes containing *hetP* and *hepA* mutated binding sites, they are much higher in the gel than the specific shifts seen from HetR bound to the native sites and likely represent nonspecific interactions between the DNA and protein. We conclude that HetR binds specifically to the *hepA* binding site and suggest that the low stoichiometric ratio of HetR to DNA used in the EMSA (2:1) may represent a physiologically relevant interaction.

The *hepA* gene encodes a component of an ABC transporter involved in the construction of the heterocyst-specific exopolysaccharide layer (31). A transcriptional fusion of the *hepA* promoter with *luxAB* showed that transcription localized to proheterocyst cells (32). To assess the function of the HetR binding site in the *hepA* promoter (P_{hepA}), a transcriptional fusion of P_{hepA} to *gfp* was constructed. Similar to the results for the published P_{hepA}-*luxAB* fusion, fluorescence from the P_{hepA}-*gfp* fusion localized exclusively to proheterocyst cells (cells committed to differentiation that have not completed morphogenesis) in the wild-type and *patA* mutant strain backgrounds, and no fluorescence was observed in a *hetR* deletion mutant (Fig. 2A and G and data not shown). The same mutation of the *hepA* binding site that abrogated specific HetR binding *in vitro* was introduced into the P_{hepA}-*gfp* fusion. With a mutated HetR binding site, no fluorescence was observed from the P_{hepA}-*gfp* fusion in any strain background under any condition tested (Fig. 2B and G and data not shown), suggesting that the interaction of HetR with the proposed binding site in P_{hepA} is necessary for transcriptional activation in proheterocysts.

HetR binds to high- and low-affinity sites *in vitro*. The master regulators of cellular differentiation in *B. subtilis*, *M. xanthus*, *S. griseus*, and *C. crescentus* have been shown to interact with specific DNA binding sites with either high or low affinities to control the timing of developmental programs (3, 6, 33, 34). To investigate the relative affinity of HetR for the *hetP* and *hepA* binding sites, the approximate K_d was determined by EMSA. The K_d is defined as the concentration of ligand (the *hetP* and *hepA* 29-bp DNA fragments) at which half is bound by protein. Since HetR interacts

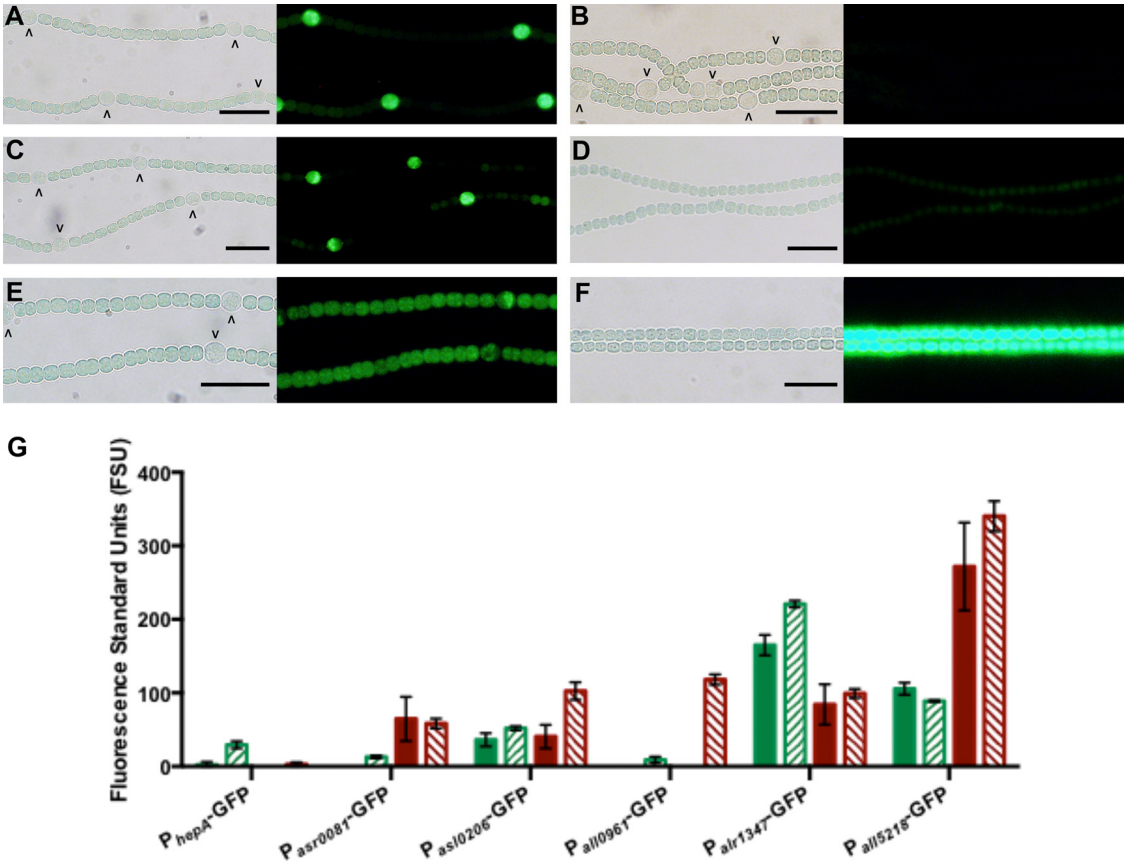

FIG 2 Qualitative and quantitative analysis of transcriptional promoter fusions to GFP. All images were obtained 24 h after the removal of combined nitrogen. (A to F) Images are light transmission micrographs (left) and fluorescence micrographs (right) of wild type (A, B, C, E) or Δ*hetR* (UHM103) (D, F) bearing P_{hepA}-GFP in pPJAV127 (A), P_{hepA}-GFP with a mutated HetR binding site in pPJAV210 (B), $P_{alr1347}$-GFP in pPJAV132 (C, D), and $P_{all5218}$-GFP in pPJAV209 (E, F). Microscope and camera settings were identical for all fluorescence micrographs. Scale bar represents 20 μm. Carets indicate heterocysts. (G) Fluorescence was quantified from the wild-type (green bars) and Δ*hetR* (UHM103) strains (red bars) bearing fusions as indicated and grown in the presence of nitrogen (solid bars) or 24 h after the removal of combined nitrogen (hatched bars). All fluorescence measurements were taken at an OD_{750} of 0.1. Error bars show standard deviations.

with DNA as a dimer, if half of the DNA is shifted, half of the protein is also bound when a 2:1 ratio of protein to DNA is used. In this assay, 200 nM *hetP* DNA was fully shifted by HetR (Fig. 3A). Using Sybr green, 100 nM DNA is nearly below the limit of detection, so the K_d of HetR binding to the *hetP* 29-bp DNA fragment *in vitro* is less than 200 nM, likely in the 100 nM range if not lower. In contrast, at least 1 μM *hepA* DNA is necessary for observable shifting, and complete shifting is not observed at even 4 μM DNA (Fig. 3B). This suggests that the K_d of HetR and *hepA* DNA is between 1 and 4 μM DNA, an order of magnitude higher than the K_d of *hetP* DNA. Taken together, these data suggest that the *hetP* 29-bp DNA fragment is a high-affinity binding site and the *hepA* 29-bp DNA fragment is a low-affinity binding site.

The *Anabaena* genome contains 166 *hepA*-like predicted HetR binding sites. As was noted by Du et al. (19), a search of the *Anabaena* genome for a portion of the *hepA* binding site yielded 29 exact matches. To identify additional sites, a BLAST search was performed using the 29-bp sequence utilized for HetR binding (35). The search found the same sites as Du et al., as well as 137 more that have a 17-bp core similar to the *hepA* binding site, totaling 166 *hepA*-like binding sites (see Dataset S1 in the supplemental material). Of these 166 sites, 85.5% (142 sites) differ from the *hepA* binding site inverted repeat sequence by two nucleotides or fewer, and 91% (151 sites) occur in intergenic regions. For sites not located within coding regions, 60% (98 sites) are between co-oriented genes, 16% (27 sites) are between convergent genes, and 15% (26 sites) are between divergent genes. These sites are found singly (66 sites) or in tandem (50 sites). In the tandem arrangement, two single binding sites are separated by 13 nucleotides in all but four cases. To determine the levels of similarity within the single and tandem groups of binding sites, phylogenetic trees were constructed. The trees for the single and tandem binding sites, constructed with 29-bp and 59-bp fragments, respectively, each branched into two main groups (see Fig. S2 and S3 in the supplemental material). To investigate the function of these sites, five single and four tandem sites were chosen, generally two from each branch of the respective tree, from promoter regions of co-orientated, probably monocistronic genes as representatives for further study.

HetR binds to single *hepA*-like binding sites *in vitro*. The ability of HetR to bind to 29-bp DNA fragments containing representative single *hepA*-like binding sites derived from the promoter regions of the genes *asr0081*, *all0961*, *all1321*, *all1748*, and *all3746* was assessed by EMSAs (Fig. 1B). A shifted species, indicating binding by HetR, was present for all five representative *hepA*-like binding sites. While the *lacZ* negative-control DNA re-

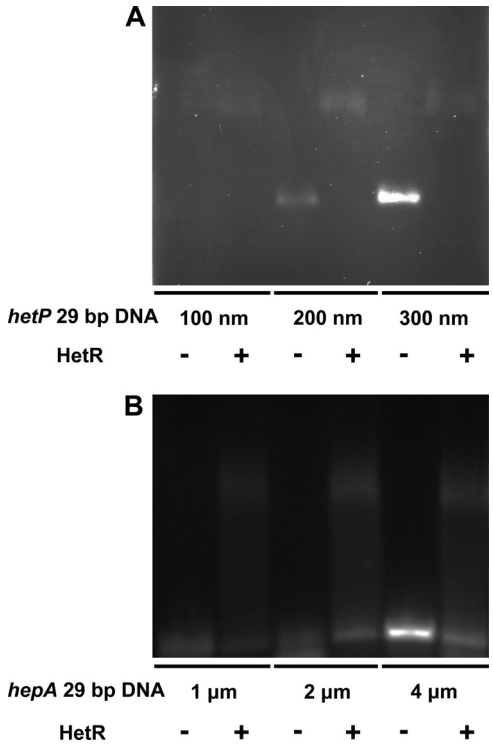

FIG 3 Electrophoretic mobility shift assays approximating the binding affinity of HetR for the *hetP* and *hepA* DNA binding sites. (A) For *hetP* 29-bp DNA fragments, the concentrations of HetR used were 100 nM (2nd lane), 200 nM (4th lane), and 300 nM (6th lane); HetR was absent from the 1st, 3rd, and 5th lanes. (B) For *hepA* 29-bp DNA fragments, the concentrations of HetR used were 1 μM (2nd lane), 2 μM (4th lane), and 4 μM (6th lane); HetR was absent from the 1st, 3rd, and 5th lanes.

mained largely unbound, the majority of the DNA from the representative fragments appears to be present in shifted bands. Additionally, the five representative *hepA*-like binding sites shifted higher in the EMSA than the *hetP* 29-bp DNA fragment. Previous research has shown that HetR binds to DNA as a dimer but can also form tetrameric species when interacting with DNA (12). It is possible that HetR binds to the five representative *hepA*-like binding sites differently than to the *hetP* binding site, and this interaction may include the multimerization of HetR.

To test the specificity of HetR interaction with the single binding sites, transversion mutations were introduced at each of the nucleotides comprising the 17-bp binding sites within the 29-bp DNA fragments containing representative single *hepA*-like binding sites, and binding was assessed (see Fig. S1 in the supplemental material). No shifted bands were observed for any of the mutated single binding sites (Fig. 1C). This indicates that the interaction of HetR with the single binding site DNA fragments requires a specific nucleotide sequence for binding rather than a general 17-bp inverted repeat motif. Together, these data show that HetR specifically binds to the five representative *hepA*-like binding sites, possibly as a tetramer, *in vitro*.

The spacing of tandem sites would not sterically hinder binding by HetR. Like the analysis of the representative single binding sites, the binding of HetR to 59-bp DNA fragments containing representative tandem binding sites derived from the promoter regions of *asl0206*, *alr1000*, *all1347*, and *all5218* was assessed by EMSA. No shifted species were observed for any of the tandem binding sites, indicating that HetR did not interact with these DNA fragments under the conditions tested (data not shown). Based on the structure of HetR bound to DNA, it appears that the flap domains that extend outwards from the DNA binding domain mediate the formation of a tetrameric complex and can interact with DNA nonspecifically (12). A model of HetR bound to a 29-bp fragment based on the *hetP* binding site showed that the majority of the nucleotides interacted with HetR, and the possibility of HetR interacting with as many as six additional nucleotides was proposed. Because the 59-bp tandem sites investigated here occur as two 29-bp binding sites with an additional base between them, it was possible that the flap domains of two HetR dimers adjacent to one another on a DNA strand could interact with each other. To test this, a synthetic 58-bp DNA fragment containing two 29-bp *hetP* binding sites aligned head-to-head was assessed for HetR binding. In an EMSA, the 58-bp fragment was shifted in the presence of HetR. The mobility of the shifted species was significantly reduced compared to the mobility of the single sites with HetR shown earlier (Fig. 1), consistent with binding of HetR to this DNA as two adjacent dimers (data not shown). Therefore, steric interference is unlikely to account for the absence of binding between HetR and the representative tandem sites.

HetR either activates or represses transcription from representative promoters. To test whether HetR binding *in vitro* is indicative of transcriptional control *in vivo*, transcriptional fusions of the nine promoter regions to *gfp* were individually introduced into the wild type, a *hetR* deletion mutant, a *hetR*(*E71N*) mutant (having an E-to-N change at position 71 encoded by *hetR*), and a *patA* mutant strain. The wild type has a normal level of HetR, the *hetR* deletion strain is devoid of HetR, and the *patA* mutant strain has been shown to produce highly elevated HetR levels (36). The *hetR*(*E71N*) mutant contains an allele of HetR in the native chromosomal locus whose protein product is unable to bind DNA *in vitro* because the E71N mutation abolishes interaction with the core of the HetR binding site consensus sequence (12). By assessing the fluorescence output from the nine transcriptional fusions at the population and individual cell levels, the effect of HetR on transcription from each promoter fusion was determined.

To account for possible plasmid copy number differences across strain backgrounds, an *rrn16Sa* promoter fusion, chosen for its robust transcription in *Anabaena* across various conditions (37), was also assessed and displayed roughly equivalent fluorescence in all strains (see Table S2 in the supplemental material). The fluorescence baseline was determined by measuring the fluorescence from each strain background containing the promoterless copy of *gfp* on pAM1956 (26). Each promoter fusion was measured in the four strain backgrounds in the presence (N^+) and absence (N^-) of combined nitrogen, and the results for representative promoter fusions are presented in Fig. 2, while the complete data set is included in the supplemental material. Quantification of fluorescence from the *asr0081*, *asl0206*, *all0961*, *alr1000*, *alr1748*, *alr3746*, and *all5218* promoter fusions generally showed that transcription was higher in both the *hetR* deletion and *hetR*(*E71N*) mutant strains and lower in the *patA* mutant strain than in the wild type; this suggests that HetR repressed transcription from these promoters (Fig. 2E, F, and G; see also Table S2 in the supplemental material). Fluorescence from *all0961* was observed only under N^- conditions, while *alr3746* was only tran-

scribed under N^+ conditions. Fluorescence from the *all1321* promoter fusion was almost entirely absent from any strain background under N^+ or N^- conditions. Unlike the other 8 promoter fusions, the *alr1347* promoter displayed higher fluorescence levels in the wild type than in either the *hetR* deletion or *hetR*(*E71N*) mutant strain, indicating that HetR contributed to the activation of this promoter (Fig. 2C and D; see also Table S2).

While the quantitative measurements described above provided a population average of transcriptional activity, at the individual cell level, GFP localization was assessed by fluorescence microscopy. Of the nine promoter fusions, only the *alr1347* promoter fusion ($P_{alr1347}$-*gfp*) displayed any heterocyst-specific transcription (Fig. 2C). In all strain backgrounds under N^+ conditions, $P_{alr1347}$-*gfp* showed low levels of fluorescence (data not shown). When nitrogen was removed, vegetative cells continued to fluoresce at a low level, while developing heterocysts fluoresced with increased intensity (Fig. 2D and data not shown). Fluorescence from all other promoter fusions was present in both cell types under N^+ and N^- conditions in all strain backgrounds tested (Fig. 2E and F and data not shown). These results suggest that HetR can function as both an activator and repressor of transcription from different promoters.

***alr1000* is required for diazotrophic growth.** To determine the involvement of the nine representative genes in diazotrophic growth, an antibiotic resistance cassette was inserted into each gene, and the growth of the mutant strains on medium lacking a source of combined nitrogen was assessed. Mutation of one gene, *all0961*, was unsuccessful, suggesting that this gene is essential. Of the eight remaining strains, only the *alr1000* mutant was incapable of growth on medium lacking a combined nitrogen source. This strain developed a wild-type pattern of heterocysts that both produced heterocyst-specific glycolipids and stained with alcian blue dye, indicating that the heterocyst exopolysaccharide layer was present (see Fig. S4 in the supplemental material). An acetylene reduction assay for nitrogenase activity showed that the *alr1000* mutant was capable of nitrogen fixation under anaerobic conditions, indicating that the heterocysts of this strain fix nitrogen only in the absence of molecular oxygen, denoting the *alr1000* mutant strain as Fox^- Fix^+ (29).

DISCUSSION

In this work, we identified an inverted repeat-containing HetR binding site in the *hepA* promoter and showed its necessity for proheterocyst-specific transcription. The *hepA* gene product is required for deposition of the exopolysaccharide layer that contributes to the physical barrier precluding oxygen entry into mature heterocysts. Strains lacking functional HepA cannot maintain the microoxic environment within heterocysts needed for the nitrogenase complex to fix nitrogen (29). Transcription of *hepA* is upregulated 5 to 10 h after the removal of combined nitrogen and localizes to proheterocyst and heterocyst cells (38, 39). Deletion analysis of the *hepA* promoter region, utilizing chromosomal *hepA*::*luxAB* fusions, identified a region required for the upregulation following nitrogen step-down located between 585 and 414 bp upstream from the translational start site (40). This region begins 7 bp upstream from a TSS that is dependent on a functional copy of *hetR* for upregulation (15) and contains the HetR binding site identified in this study. Smaller deletions in this region showed that removal of the HetR binding site resulted in a modest reduction in transcription (40); however, our data show a complete loss of *gfp* fluorescence when the HetR binding site was mutated, indicating abrogation of transcription. While these studies both show an effect on *hepA* transcription from the loss of the HetR binding site, differences in the reporters and their placement on plasmids or in the chromosome could account for the discrepancy.

The site in *hepA* was used to identify 166 potential HetR binding sites that were similar in sequence. This number of potential HetR binding sites is comparable to the number of binding sites in other well-studied regulons, including the cyclic AMP receptor protein (CRP) (41), RpoN (42), and PhoP/Q (43) regulons in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Yersinia pestis*, respectively, as well as the developmental regulons of Spo0A from *Bacillus subtilis* (2) and CtrA from *Caulobacter crescentus* (8). Of the 166 sites, only 13 *hepA*-like sites were tested, and evidence consistent with real binding of HetR with the single sites was found. While only a subset were tested, three lines of evidence suggest that the *hepA*-like binding sites are biologically relevant and are not present throughout the *Anabaena* genome by chance. First, taking into account the 41.2% GC content of the *Anabaena* genome, the 17-bp core *hepA* sequence is predicted to occur less than once. Instead, this 17-bp sequence occurs 37 times, 68 times if either member of the A/T pair in the middle of the inverted repeat sequence is allowed. Second, the majority of the *hepA*-like binding sites are located in intergenic regions. Of the 166 *hepA*-like binding sites, 91% are present in intergenic regions, compared to 17.4% of the genome that is predicted to not code for protein (44). The HetR binding sites located in open reading frames may regulate transcription, as work on the *E. coli* RutR regulon showed that the majority of its binding sites were located within coding regions (45). Third, nucleotide sequences similar to the *hepA* binding site are present in heterocystous cyanobacteria but absent in a nonheterocystous strain. A BLAST search of the 17-bp *hepA* palindrome revealed 20 similar sites in *Nostoc punctiforme* ATCC 29133 and *Anabaena azollae* 0708, 25 in *Anabaena cylindrica* PCC 7122, 149 in *Anabaena variabilis* ATCC 29413, all of which form heterocysts, and none in *Trichodesmium erythraeum* IMS101, a filamentous, nonheterocystous nitrogen-fixing strain. *Trichodesmium* can grow diazotrophically, but nitrogen fixation relies on a fluctuating ratio of temporally regulated diazocytes rather than on a fixed ratio of spatially regulated heterocysts (46, 47). The lack of heterocysts could account for the lack of *hepA*-like HetR binding sites in *Trichodesmium*. These data are consistent with the biological relevance of the proposed HetR binding sites.

The interaction of HetR with the binding site present in the *hetP* promoter is the best-characterized example of HetR binding DNA to date. In EMSAs, HetR was shown to interact with the *hetP* binding site at a 1:1 HetR dimer/DNA ratio, and a supershifted species was observed at a 2:1 HetR dimer/DNA ratio, indicative of a tetrameric HetR complex (22). This HetR-DNA interaction was abrogated by mutation of six bases within the 17-bp binding site palindrome (18). Additionally, crystal structures of HetR bound to DNA based on the *hetP* binding site elucidated the specific contributions of the amino acid residues that facilitate the interaction of HetR with DNA and described HetR binding to DNA as a tetramer (12). In the work presented here, the K_d estimation of HetR for the *hetP* and *hepA* binding sites showed that HetR interacted with the *hetP* binding site with a higher affinity than with the *hepA* site. Based on these differences in affinity and published

stoichiometric ratios used in EMSAs of HetR with additional known sites, we propose that HetR interacts with three categories of binding sites to regulate different phases of heterocyst differentiation: category I is comprised of sites that have been difficult to delimit and define and exhibit low binding affinity *in vitro*; category II is comprised of well defined sites that share a similar inverted repeat sequence and exhibit an intermediate level of binding affinity *in vitro*; and category III is comprised of well defined sites that share a similar inverted repeat sequence different from the category II sites and exhibit a high level of binding affinity *in vitro*.

Category I HetR binding sites are poorly defined. Large DNA fragments, labeled with ^{32}P or biotin, from the promoters of *hetR* (16, 17, 48), *patS* (16), and *pknE* (17) have been shown to interact specifically with HetR in EMSAs. However, stoichiometric ratios ranging from 50:1 to 1,500,000:1 of HetR to labeled DNA were required for shifting. Binding of the P_{hetR} DNA fragment at a 50:1 ratio did not result in complete shifting (17), and shifting of the entire band has only been observed at far higher stoichiometric ratios (48). Binding assays utilizing the method in this study were unable to shift fragments derived from the *hetR* promoter (S. Ni and M. A. Kennedy, unpublished results). This method is not amenable to large stoichiometric ratios, so a direct comparison is not possible.

Transcription of the *hetR*, *patS*, and *pknE* genes is upregulated by 0.5 (49), 3 (50), and 3 h (17), respectively, following the removal of combined nitrogen and localizes to single cells roughly 6 to 8 h following upregulation. All three genes require a functional copy of *hetR* for upregulation (16, 17, 49), but sequence determinants that provide specificity for HetR binding to these promoters are unknown. Potential HetR binding sites derived from the *hetR* and *patS* promoters were tested for interaction in this work by performing EMSA, but no binding was observed (data not shown). It is possible that additional proteins absent from the EMSAs may interact with HetR to mediate binding or that post-translational modification of HetR may facilitate binding to category I sites. As the upregulation of *hetR*, *patS*, and *pknE* coincides with the patterning phase of differentiation (Fig. 4), additional factors required for high-affinity binding *in vitro* may also be upregulated during this phase.

The binding sites found in the work described here fall into category II, which consists of sites with a defined palindromic sequence and a binding affinity *in vitro* that falls between that of category I and III sites. Many category II binding sites are adjacent to genes without a clear connection to heterocyst function but which may be involved in vegetative cell metabolism or heterocyst morphogenesis. Metabolism under nitrogen-limiting conditions is inherently different from nitrogen-replete growth in that fixed nitrogen is provided exclusively from heterocysts rather than imported from the environment. A role for HetR as a general regulator of metabolism or heterocyst morphogenesis in the absence of nitrogen is consistent with the overexpression of *hetP* in a *hetR* deletion mutant resulting in the formation of heterocysts that were only capable of nitrogen fixation under anoxic conditions (18). It is possible that, while morphologically distinct heterocyst cells can form in the absence of *hetR* with overexpression of *hetP*, certain functional components are lacking that would normally be controlled by HetR. This finding is consistent with the *arl1000* mutant described in this work that, although it was able to produce morphologically distinct heterocysts, only fixed nitrogen under anoxic conditions.

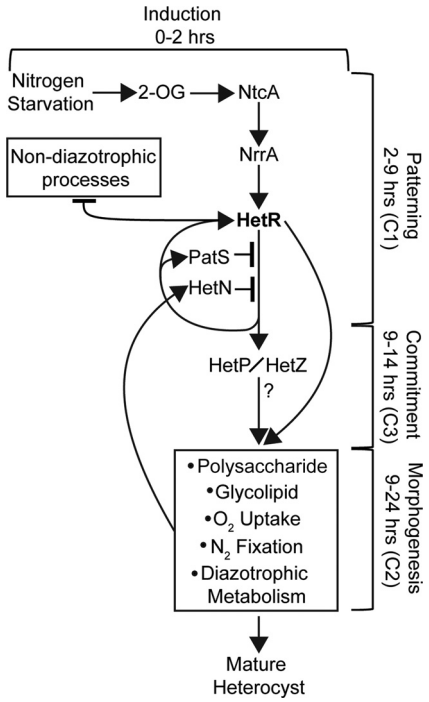

FIG 4 A model depicting the genetic interactions involved in the developmental cascade of heterocyst differentiation. The time during which the components of each phase function following the removal of combined nitrogen is indicated. Arrows indicate positive interactions, while bars indicate negative interactions. C1, C2, and C3 correspond to the category I, II, or III HetR binding sites, respectively, thought to contribute to the phase of differentiation. Non-diazotrophic processes refers to processes that are downregulated during diazotrophic growth.

Category III HetR binding sites have inverted repeat sequences with a low K_d and high binding affinity *in vitro*. Like category II sites, mutation of nucleotides within the 17-bp inverted repeat abrogates HetR binding (18). The only two category III binding sites, those present in the *hetP* and *hetZ* promoters, are shifted at 1:1 and 6:1 HetR dimer-to-DNA ratios, respectively (22; P. Videau and S. M. Callahan, unpublished results). The *hetP* and *hetZ* genes have been reported to have overlapping function, and overexpression of *hetP* can partially bypass *hetR* (18, 51). It has been proposed that HetP acts as a slave to HetR such that HetP, and perhaps HetZ, executes the patterning decision made by HetR and PatS. Consistent with this hypothesis, the functions of *hetP* and *hetZ* and the timing of their upregulation coincide with the commitment phase of differentiation (Fig. 4) (39).

Recent work in *Anabaena* identified TSSs in the wild type and a Δ*hetR* mutant during growth with a source of nitrogen and 8 h following nitrogen removal (15). A set of TSSs, termed DIF$^+$ (differentiation-related changes in transcription from TSSs in the wild type), were upregulated >8-fold in wild-type *Anabaena* but not in a Δ*hetR* mutant. Of the category II HetR binding sites identified in this work, only 4 promoters with category II sites contain TSSs in the DIF$^+$ category; these TSSs are within the promoter regions of *all0284*, *alr2835*, *alr3554*, and *alr4919*. The TSSs nearest to the remaining category II sites did not show an obvious correlation with regulation by HetR. The distances of category II sites to

the closest TSS were fairly evenly distributed, but this could be an artifact of an incomplete data set. The binding sites presented here are part of an initial characterization of the direct HetR regulon and probably do not encompass all HetR binding sites.

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