Expression of *hetN* during heterocyst differentiation and its inhibition of *hetR* up-regulation in the cyanobacterium *Anabaena* sp. PCC 7120

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Abstract **The *hetN* gene plays an important role in heterocyst differentiation and pattern formation. An immunoblotting study showed that the *hetN* gene in *Anabaena* sp. PCC 7120 was expressed in vegetative cells grown with combined nitrogen. After a switch to a medium without combined nitrogen, *hetN* expression first declined and was then followed by a rapid increase in its product, HetN, which was only present in mature heterocysts. HetN is located on both thylakoid membranes and plasma membranes as determined by immunoblotting using purified membranes. Overexpression of *hetN* completely prevented *hetR* up-regulation under nitrogen-deprivation conditions, suggesting that its role in pattern control may depend on its inhibition of *hetR* expression.**

Key words: Fatty acid; Heterocyst; Pattern formation; Polyketide

1. Introduction

Cyanobacteria are a diverse group of oxygenic photosynthetic prokaryotes. Some filamentous cyanobacteria can form differentiated cells, called heterocysts, for nitrogen fixation under nitrogen-deprivation conditions [1,2]. Heterocysts differ from vegetative cells in several aspects [3]. They have a thick envelope to limit oxygen penetration into cytoplasm, and a higher respiratory activity to consume oxygen as well, to provide ATP for nitrogen fixation.

In many heterocystous cyanobacteria, heterocysts are regularly spaced so that a pattern is present along the filaments. The *hetR* gene is essential for heterocyst differentiation and pattern formation [4,5]. It encodes a serine-type protease with autodegradative activity [6,7], and its expression is positively autoregulated [8]. Two important genes that have been shown to suppress heterocyst differentiation are *patS* and *hetN*. The *patS* gene encodes a short peptide that can be further processed to an even smaller peptide to suppress heterocyst differentiation. When the pentapeptide RGSGR, which occurs at the C-terminus of PatS, is externally added to the growth medium, it fully prevents formation of heterocysts [9]. The *hetN* gene was first reported by Black and Wolk [10] and Bauer et al. [11]. Although the exact product of HetN remains unknown, HetN has a high sequence similarity to ketoacyl reductase, which is involved in fatty acid or polyketide synthesis. In a *hetN* insertion mutant strain, several phenotypes were observed including Het^- and multiple contiguous heterocysts (Mch), and the authors speculated it could be second site mutations that caused these phenotypes [10]. Northern analysis showed that the *hetN* mRNA could be detected 12 h after the removal of combined nitrogen. Recently, Callahan and Buikema [12] constructed an *Anabaena* sp. PCC 7120 (*Anabaena* 7120) strain such that the *hetN* was under control of the copper-inducible *petE* promoter. Their results showed that overexpression of *hetN* prevented a patterned expression of *hetR*. On the other hand, the lack of HetN production in the absence of copper in the growth medium resulted in a delayed Mch phenotype. They also showed that HetN is primarily located in heterocysts and suggested that HetN was required for the pattern maintenance. However, one question remains unanswered: if *hetN* is only expressed in heterocysts, why does a *hetN* null mutant produce second site mutations under nitrogen replete conditions?

The mechanism of inhibition of heterocyst differentiation by PatS and HetN is not understood. In a search of the *Anabaena* 7120 genome, we found an RGSGR motif, which is the bioactive peptide present at the C-terminus of PatS, within the primary sequence of HetN. Mutagenesis was performed to analyze the role of this motif in HetN, and the results are reported in this paper. Here we report the results of immunoblotting studies of HetN as well as the effect of *hetN* overexpression on HetR.

2. Materials and methods

2.1. Strains and culture conditions

Anabaena sp. PCC 7120 was grown in BG-11 medium [13] with or without $NaNO_3$ at 28°C under illumination with cool white fluorescent light. Neomycin was added to the medium at a concentration of 50 μg ml^{-1} when needed. *Escherichia coli* strains were grown in LB medium. Strain DH5α was used for all routine cloning purposes. Strain BL21(DE3) was used for overproduction of recombinant HetN. Strain HB101 was used for conjugal transfer of plasmids from *E. coli* to *Anabaena* 7120 [14].

2.2. Plasmid construction and site-specific mutagenesis

The plasmid for overexpressing the *hetN* gene from *Anabaena* 7120 in *E. coli* was constructed as follows. The coding region of *hetN* was amplified with polymerase chain reaction (PCR) using the following primers: 5′-TACCATGGCAACTCTTACAGGTAAGACAG-3′ and 5′-TTGGATCCGATCTGATCAAAACCTCATTCC-3′ in the presence of Pfu DNA polymerase [15]. The PCR product was cloned

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Abbreviations: Mch, multiple contiguous heterocysts; PCR, polymerase chain reactions; rHetN, recombinant HetN; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis

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into vector pGEM-T (Promega). The resultant plasmid was digested with *Bam*HI and *Nco*I and the DNA fragment was then ligated into pET3d [16] to generate pET3dhetN so that *hetN* was transcribed by T7 RNA polymerase upon induction. This construct was confirmed by DNA sequencing. The plasmids carrying the wild-type and mutant *hetN* genes were constructed as follows. The coding region of the *hetN* plus 600 bp upstream of the start codon was amplified by PCR using the primers 5′-TTGGATCCATGACCTACACAGCTTACTTGAT-3′ and 5′-TTGGATCCGATCTGATCAAAACCTCATTCC-3′. The DNA fragments obtained were cloned into vector pGEM-T to generate pT-hetN, which was used as a template for site-specific mutagenesis (see Section 3.3). Each *hetN* gene with its promoter region was then digested with *Bam*HI and cloned into pRL25C [17]. The resultant plasmids, which have their *hetN* genes in the same transcription direction as the neo^R gene on pRL25C, were transferred into *Anabaena* 7120 by triparental conjugation [14].

To perform site-specific mutagenesis of the RGSGR motif, the long inverse PCR method [18] was used with the entire pT-*hetN* plasmid as a template. The primer 5′-TTGTTAACATTGCTTCTTTAGCTGGTAAAAAGGG3′ was paired with primers 5′TTGTTAACAATCCGACCACTACCCTTTTCCATC-3′, 5′-TTGTTAACAATCCGACCATCACCGCGTTCCATC-3′, 5′-TTGTTAACAATCCGACTACTAC-CGCGTTCCATC-3′ and 5′-TTGTTAACAATCAGACCACTACCGCGTTCCATC-3′ in PCR to generate hetNR132K, hetNS134D, hetNG135S and hetNR136L, respectively. All PCR products were digested with *Hpa*I and ligated before being transformed into *E. coli* cells. All mutations generated were confirmed by DNA sequencing. The plasmids with mutant *hetN* genes were digested with *Bam*HI and cloned into pRL25C.

2.3. Immunoblotting

The induction of *hetN* by the removal of combined nitrogen was studied as follows. The *Anabaena* 7120 filaments grown with $NaNO_3$ were washed three times with HEPES buffer and incubated in BG-11 lacking combined nitrogen at a chlorophyll concentration of 2 μg ml^{-1} under illumination. The cells were collected at intervals as indicated in the text and total cellular extracts were prepared by French press treatment of the cells at 168 MPa. The proteins were separated by SDS–PAGE and transferred to a polyvinylidene difluoride membrane electrophoretically. The antibodies against either rHetN or rHetR were used as primary antibodies in immunoblotting, and the rest of the procedures was performed according to Zhou et al. [19]. Quantification of proteins after immunoblotting was determined with ImageMaster VDS software (Pharmacia, Hong Kong, PR China). HetN localization was performed with isolated thylakoid membranes and plasma membranes as described in [20,21]. The purity of the isolated membranes was determined with anti-PsaE antibodies according to Norling et al. [20].

2.4. Other methods

The N-terminal sequence of rHetN was determined with an ABI Procise sequencer as described in[6]. Heterocyst isolation was performed according to the following method [19]. The filaments were passed through a French press three times at a pressure of 80 MPa and centrifuged at $600 \times g$ for 5 min. The supernatant is the vegetative cell fraction. The pellet containing heterocysts was washed three times by centrifugation to remove large cell debris. Amino acid and DNA sequence analyses were performed with DNAsis software (Hitachi, Hong Kong, PR China). Antibody purification was performed with the antigen affinity purification method [22].

3. Results

3.1. Immunoblotting study of the expression of hetN in Anabaena 7120

In order to understand the biochemical function of HetN, the *hetN* gene from *Anabaena* 7120 was overexpressed in *E. coli*. Recombinant HetN (rHetN) accumulated as inclusion bodies upon induction. The inclusion bodies were dissolved with 7 M urea and purified by gel filtration followed by gel purification with SDS–PAGE separation (Fig. 1). Antibodies against rHetN were raised in rabbits.

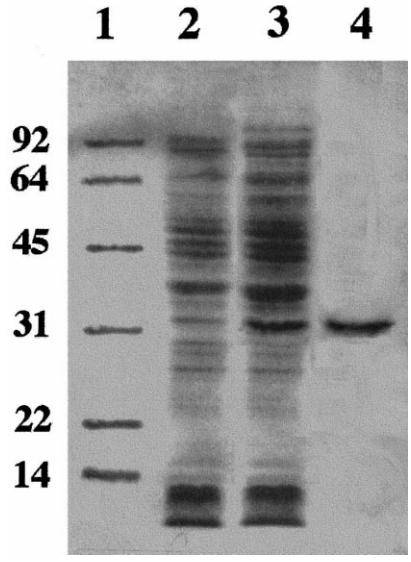

Fig. 1. Overexpression of the *hetN* gene from *Anabaena* 7120 in *E. coli*. Lanes 2 and 3, *E. coli* strain BL21(DE3) containing pET3d-hetN without and with induction, respectively. Lane 4, purified rHetN as confirmed with N-terminal sequencing. The molecular mass standard proteins are in lane 1 with their masses in kDa shown on the left side.

Fig. 2A shows the results of the expression of the *hetN* gene from *Anabaena* 7120, as studied by immunoblotting with total cellular extracts, during heterocyst differentiation induced by shifting a culture from a medium with combined nitrogen to a nitrogen-deprived condition. A protein with an apparent mass of 31 kDa was detected (lane 2) in vegetative cells grown in the presence of combined nitrogen. The amount of HetN during heterocyst differentiation first declined before it increased to a level higher than the original level. The amount of HetN in filaments 3 h after shifting to the nitrogen-depleted condition was similar to the initial level (lane 3), and it was reduced approximately by 60% (lane 4) in 6 h and was further reduced to 20–30% of the original level (lane 5) under nitrogen depletion condition. The amount of HetN in the filaments containing mature heterocysts, 24 h after the induction, was about three times higher than the original level (lane 6). Immunoblotting was also performed with cellular extracts from isolated vegetative cells and heterocysts of the filaments grown without combined nitrogen, and these results are shown in Fig. 2B. HetN was only detected in isolated heterocysts, while little HetN was observed in the isolated vegetative cells from the filaments. We estimate that the HetN concentration in heterocysts is between 0.5% and 1% of the total cellular protein; this is 30 times higher than the average concentration of HetN in the vegetative cells grown with combined nitrogen. In both Fig. 2A and B, there is a band of higher molecular mass that cross-reacts with the anti-rHetN antibodies in the heterocyst-containing fractions. The nature of this band has not been studied further.

3.2. Localization of HetN in Anabaena 7120

Homology search results suggested that HetN could be involved in fatty acid synthesis [10]. However, it is not entirely clear whether HetN is a membrane protein or a cytoplasmic protein based upon its primary amino acid sequence. To study the localization of HetN in *Anabaena* 7120, immunoblotting

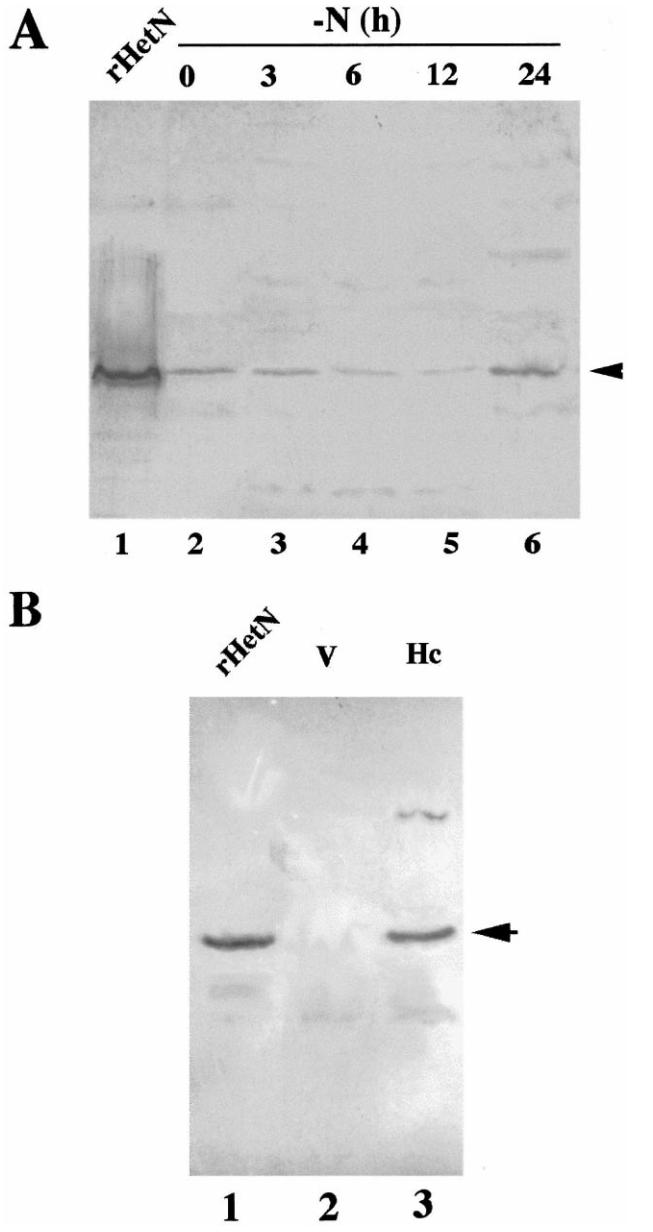

Fig. 2. Immunoblotting studies of *hetN* expression and the distribution of HetN. A: *hetN* expression during heterocyst differentiation. *Anabaena* 7120 filaments grown with combined nitrogen were shifted to a nitrogen-deprived condition and total cellular extracts at various times (as shown in hours at the top of the figure) after the shift were analyzed. B: Determination of HetN distribution between heterocysts (Hc) and vegetative cells (V) from the filaments grown without combined nitrogen. Approximately 100 μg proteins from heterocysts and vegetative cells and 0.5 μg rHetN were loaded. Arrows indicate the position of HetN.

was performed with isolated thylakoid and plasma membranes, and the results are shown in Fig. 3. While HetN was detected in both types of membranes, the amount of HetN on thylakoid membranes was approximately three to five times higher than that associated with the plasma membranes. The results show that most HetN is tightly associated with membranes. This is consistent with the suggestion that HetN is involved in fatty acid synthesis. A faint band is also detected in lane 1 of Fig. 3, indicating that a small fraction of HetN could be in the soluble, cytoplasmic fraction.

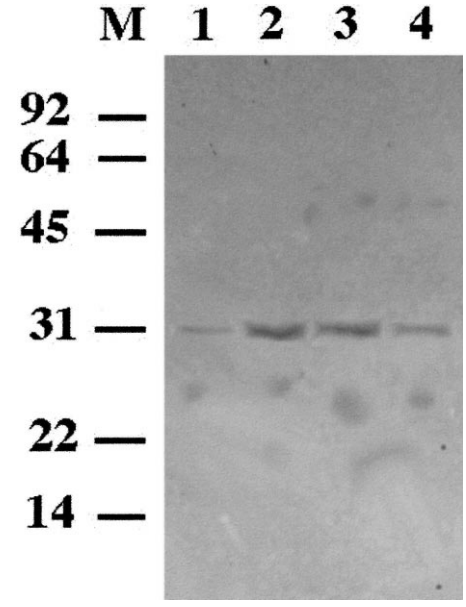

Fig. 3. Subcellular localization of HetN. Proteins from cytoplasmic fraction (lane 1), total membranes (lane 2), thylakoid membranes (lane 3) and plasma membranes (lane 4) were separated with SDS–PAGE and analyzed for the presence of HetN with immunoblotting. Approximately 100 μg protein was loaded in each lane. The molecular mass standards are shown on the left side (in kDa).

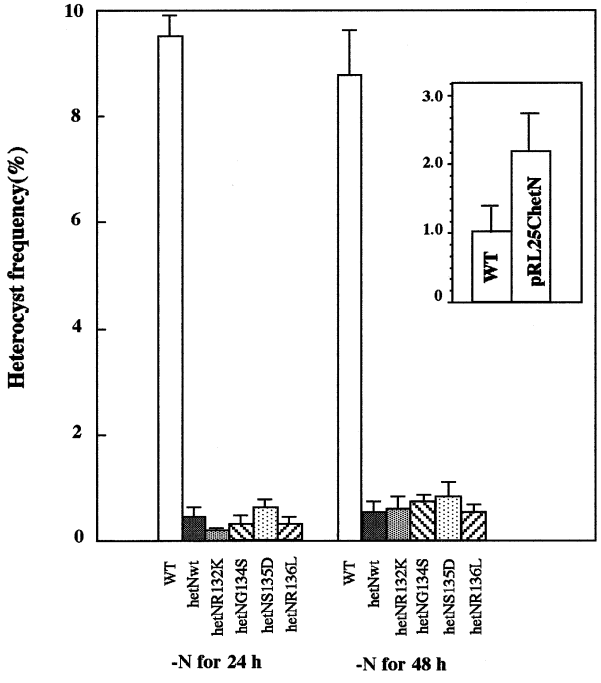

Fig. 4. Suppression of heterocyst differentiation by overexpression of *hetN* and its mutants. *hetN* and the four mutant *hetN* genes as shown were placed on pRL25C and introduced into *Anabaena* 7120. The heterocyst frequency (%) was counted 24 h and 48 h after shifting the culture to a condition without combined nitrogen. Each point represents the average of heterocyst frequencies from three different cultures. The heterocyst frequency was determined by counting along the filaments of *Anabaena* 7120. Inset: Relative amount of HetN in the wild-type strain (WT) and filaments with multiple copies of *hetN* on pRL25C (pRL25C*hetN*) as determined by immunoblotting. Three separate blots were used to determine the amount of HetN.

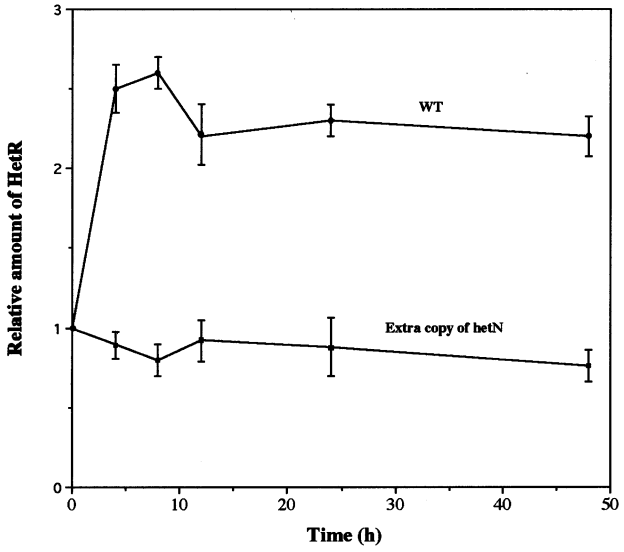

Fig. 5. Effect of overexpression of *hetN* on *hetR* gene expression. *Anabaena* 7120 filaments grown with combined nitrogen were shifted to a nitrogen-free medium and the amount of HetR in total cellular extracts at various times after the shift was analyzed by immunoblotting. All measurements were normalized against the amount at the zero hour and each point was the average of three separate blots.

3.3. Site-specific mutagenesis analysis of HetN

In a search of peptide patterns in the *Anabaena* 7120 genome, we found that HetN contains an RGSGR motif from position 132 to 136, which is identical to the bioactive, C-termial pentapeptide of PatS. Since the PatS peptide and HetN have similar roles in pattern formation in *Anabaena* 7120 and since the pentapeptide at the C-terminus of PatS is sufficient to inhibit heterocyst differentiation [9], the finding of an RGSGR motif in HetN suggested that this could be important in the inhibition of heterocyst differentiation by HetN. Site-specific mutagenesis was performed to change these amino acid residues, and the mutant *hetN* genes were introduced into wild-type *Anabaena* 7120 on the multicopy plasmid pRL25C to see if they would inhibit heterocyst differentiation as the wild-type *hetN* gene does. Fig. 4 shows the changes of heterocyst frequencies of *Anabaena* 7120 with pRL25C containing the wild-type and mutant *hetN* genes after shifting to the nitrogen depletion condition. Very few heterocysts were observed 24 h after induction in the *Anabaena* 7120 strain with pRL25C*hetN* as compared to the wild-type *Anabaena* 7120, which had a heterocyst frequency of 9%. Some heterocysts could be observed in the *Anabaena* 7120 strain containing pRL25C*hetN* 48 h after the removal of combined nitrogen, and their frequency was approximately 1%. No further increase of heterocyst frequency was observed upon further incubation without combined nitrogen. Fig. 4 also shows that multiple copies of *hetN* in *Anabaena* 7120 resulted in overproduction of HetN (inset) as demonstrated by a 1.2-fold increase of HetN in filaments containing pRL25C*hetN*.

The four mutations introduced in the RGSRG motif in the *hetN* gene were: R132K, G134S, S135D and R136L. The inhibition of heterocyst differentiation by the mutant *hetN* genes introduced on pRL25C is also shown in Fig. 4. Similar to the wild-type *hetN*, all of these mutant *hetN* genes on the multiple copy plasmid suppressed heterocyst differentiation and reduced heterocyst frequencies in *Anabaena* 7120 under the nitrogen depletion condition. The strains carrying the four mutant *hetN* genes on pRL25C also had a one- to two-fold increase of HetN amount in their cell extracts (data not shown). These results showed that the role of HetN in heterocyst differentiation and pattern formation was not due to the presence of the RGSGR peptide motif.

3.4. Inhibition of hetR induction by HetN

The delay and inhibition of heterocyst formation shown in Fig. 4 suggested that HetN inhibited the early steps in the differentiation process. It has been shown that the *hetR* gene is one of the earliest genes induced by nitrogen step-down [4,19]. The effect of the *hetN* gene present on the multiple copy plasmid pRL25C on *hetR* gene induction was studied with immunoblotting (Fig. 5). It is evident that the *hetR* gene in *Anabaena* 7120 carrying pRL25C*hetN* had no up-regulation. The amount of HetR protein was almost unchanged 24 h after nitrogen step-down and the amount of HetR was only 40% of that in the wild-type filaments 48 h after nitrogen step-down.

4. Discussion

While overexpression of *hetR* results in an Mch phenotype [4,5], overexpression of *patS* and *hetN* leads to suppression of heterocyst differentiation [10,12]. PatS and HetN seem to play similar roles in the suppression of heterocyst formation and in pattern formation [9,12]. The presence of a PatS C-terminal pentapeptide within the HetN protein primary sequence initially suggested to us that this motif in HetN could be critical to its biochemical function(s). However, mutagenesis analysis of the *hetN* gene showed that this motif is not involved in its function of heterocyst suppression (Fig. 4). These studies also ruled out the possibility that the inhibition of heterocyst differentiation by HetN was due to the RGSGR pentapeptide generated as a proteolytic product of HetN.

The results of HetN localization (Fig. 3) show that it is membrane-associated and support the suggestion that HetN might be involved in fatty acid synthesis or polyketide synthesis [10], which is important in heterocyst formation in another heterocystous cyanobacterium, *Nostoc punctiforme* [23]. Since the DNA fragment containing *hetN* and used for overexpression (Fig. 4) did not include *hetI*, which is transcribed from the opposite direction, our results show that *hetN* itself is sufficient to suppress heterocyst differentiation, in agreement with the results from Callahan and Buikema [12]. The localization experiments based on a green fluorescence protein reporter protein [12] and immunoblotting in this study (Fig. 2) show that HetN is located in the heterocyst when *Anabaena* 7120 is growing in a medium without combined nitrogen. Because HetN is too large to diffuse into neighboring vegetative cells, the inhibitory effect of HetN on heterocyst formation around a mature heterocyst is likely due to its enzymatic product as suggested [10].

The expression of *hetN* during heterocyst differentiation, as studied by Northern analysis [11] and immunoblotting (Fig. 2), showed that the *hetN* gene was up-regulated by the removal of combined nitrogen from the growth medium. However, the immunoblotting study also shows that HetN is present in vegetative cells of the *Anabaena* 7120 filaments grown in a me-

dium with combined nitrogen (Fig. 3). The amount of HetN in vegetative cells grown with combined nitrogen was very low, and this could be the reason why Northern analysis did not detect *hetN* mRNA in filaments grown with combined nitrogen [11]. When *hetN* was initially studied, the *hetN* mutant showed several phenotypes, including loss of heterocysts and Mch [10]. When the *hetN* gene is under the control of a copper-inducible promoter, no heterocysts are formed when combined nitrogen is present in the growth medium, even in the absence of copper induction [12]. The Mch phenotype could only be observed 48 h after removal of combined nitrogen when *hetN* was not induced with copper. These observations and the results shown in this report suggest that HetN might be required for suppression of heterocyst formation when combined nitrogen is present. The basal expression level in the strain carrying copper-inducible *hetN* [12] could be sufficient to suppress heterocyst differentiation under nitrogen replete conditions. When the *hetN* gene is inactivated, no HetN is available to inhibit heterocyst differentiation and secondary mutations could occur in the *hetN* mutant strain [10,12]. Our results indicate that the HetN level in proheterocysts is not high based on immunoblotting 12 h after nitrogen step-down when proheterocysts are present (Fig. 2). The inhibition of *hetR* expression (Fig. 5) by HetN indicates that HetN functions early in the differentiation process. This could also explain why *hetN* is up-regulated only 12 h after the removal of combined nitrogen, since early induction of *hetN* could prevent heterocyst differentiation completely. With recombinant HetN available, it should be possible to study HetN's biochemical function in order to understand its roles in heterocyst differentiation and pattern formation.

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