HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS

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HetR plays a key role in regulation of heterocyst differentiation. When the Cys-48 residue of the HetR from *Anabaena* sp. PCC 7120 was replaced with an Ala residue, the mutant HetR ($HetR_{C48A}$) could not dimerize, indicating that HetR forms a homodimer through a disulfide bond. The *Anabaena* strain C48, containing the *hetRc48a* gene, could not produce HetR homodimer and failed to form heterocyst. We show that HetR is a DNA-binding protein and that its homodimerization is required for the DNA binding. HetR binds the promoter regions of *hetR*, *hepA*, and *patS*, suggesting a direct control of the expression of these genes by HetR. We present evidence that shows that the up-regulation of *patS* and *hetR* depends on DNA binding by HetR dimer. The pentapeptide RGSGR, which is present at the C terminus of PatS and blocks heterocyst formation, inhibits the DNA binding of HetR and prevents *hetR* up-regulation.

The heterocystous cyanobacteria such as *Anabaena* sp. PCC strain 7120 contain specialized cells called heterocysts for nitrogen fixation when they are grown in the absence of combined nitrogen (1, 2). Many structural and metabolic changes occur during heterocyst differentiation (2–5). In cyanobacteria with long filaments, the spacing of heterocysts along the filaments is often regular, so there is a pattern formation (1). According to fossil records, the heterocyst pattern was one of the earliest pattern forms in evolution (6, 7).

The pattern formation of cyanobacteria depends on cell–cell communication and molecular interactions (1, 8). Several important genes, such as *ntcA* (9, 10), *hetR* (11), *hetC* (12), *hetF* (13), *hetN* (14, 15), and *patS* (16, 17), play important roles in heterocyst differentiation and pattern formation. Recent evidence suggests that α-ketoglutarate could play an important role in the initiation of heterocyst differentiation (18).

hetR is the master gene in controlling heterocyst differentiation and pattern formation (3, 4, 19), and it may also control other cellular processes in nonheterocystous cyanobacteria (20). By putting *hetR* under the control of the copper-inducible promoter PpetE, it was shown that heterocyst frequency was controlled by the expression level of *hetR* (19). *hetR* is autoregulatory (21) and regulates the expression of some other genes involved in heterocyst differentiation (8, 21). HetR is a Ser-type protease required for heterocyst differentiation (22, 23). The mechanism for regulation of heterocyst differentiation by HetR is not clearly understood. The study by Buikema and Haselkorn (19) raised a very interesting question: Why does HetR as a protease function in a dose-dependent fashion?

patS encodes a small peptide, and a diffusible shorter peptide could be generated from the full gene product (16, 17). Like *hetR*, the expression of *patS* is localized primarily in proheterocysts and heterocysts (16). However, little is known about the regulation of the *patS* expression and the molecular mechanism for inhibition of heterocyst differentiation by PatS.

In this communication, we report that HetR functions as a homodimer *in vivo*. We show that HetR is a DNA-binding protein and that dimerization of HetR is required for its DNA-binding activity and heterocyst differentiation. We also show that the PatS peptide inhibits the DNA-binding activity of HetR.

Materials and Methods

Strains and Growth Conditions. *Anabaena* sp. PCC 7120 and its related strains were grown in BG11 or $BG11_0$ media as described (23, 24). *Escherichia coli* was grown in LB medium at 37°C. The strain DH5α was used for all general cloning purposes. The strain BL21(DE3) was used for overproducing proteins (25).

DNA Manipulation. Site-specific mutation of the *hetR* gene of *Anabaena* sp. PCC 7120 was performed as described (23). An oligonucleotide (5′-ACGGCGGCTAAGGCTGCCATTTA-CATG-3′) was used for C48A mutation, and the resultant mutant gene was confirmed by DNA sequencing and named *hetRc48a*. The *hetR* and *hetRc48a* genes were introduced to the strain 884a (21) as described in ref. 23 to generate strain WT211 and strain C48, respectively. The strain WT211 is identical to the wild-type strain in growth rate, *hetR* induction, and heterocyst pattern formation (23).

Isolation of total cyanobacterial genome DNA and total RNA was performed according to Zhao *et al.* (26). An internal fragment of the *hetR* gene (+101 to +509) was amplified by PCR and used as template for synthesizing the radioactive probe for both Southern and Northern hybridizations. The primers used for the PCR amplification were 5′-GGCATGGAGCATTCT-TAG-3′ and 5′-AGATCCTCTTGCGATCGC-3′. The probe was generated with [α-^{32}P]dCTP (111TBq·mmol^{-1}) with a random primer DNA labeling kit (Takara, Beijing).

Yeast Two-Hybrid Analysis. The MATCHMAKER Two-Hybrid Kit (Clontech) and the *Saccharomyces cerevisiae* strain Y187 were used for yeast two-hybrid assays. The wild-type *hetR*, *hetRc48a*, and *hetRs179n* genes were PCR-amplified by using the primers 5′-AACTAGGATCCATATGAGTAACGACATC-3′/5′-AACTGGATCCGC TTAATCTTCTTTTCTA-3′ and 5′-AACTAGGATCCATATGAGTAACGACATC-3′/5′-AAC-TCTGCAGGCTTAATCTTCTTTTCTA-3′ and cloned in the pACT2 and pAS2-1 plasmids (Clontech), respectively. For the β-galactosidase colony-lift assay, each transformant was grown on an agar plate at 30°C for 2–3 days. Whatman paper filters were placed over the surface of the plates. The wetted filters with colonies were lifted off the agar plates and subjected to three cycles of freezing and thawing between liquid nitrogen and room temperature. The filters were then placed onto other filter papers presoaked with buffer Z (60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgSO_4$, pH 7.0) containing 250 μg·ml^{-1} 5-bromo-4-chloro-3-indolyl-D-galactopyranoside and incubated at 30°C for detecting galactosidase activity by observ-

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Abbreviations: DnsF, Dansyl fluoride; EMSA, electrophoretic mobility-shift assay.

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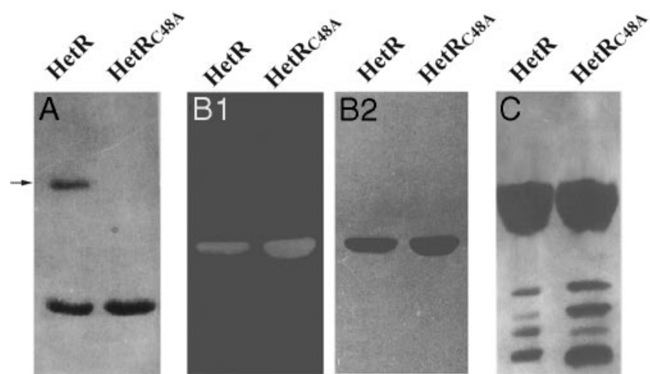

Fig. 1. HetR$_{C48A}$ is unable to form homodimer. (*A*) SDS/PAGE analysis of the HetR proteins under a nonreducing condition. The gel was stained with Coomassie blue. HetR dimer is indicated by the arrow. (*B1* and *B2*) DnsF labeling of the HetR proteins. DnsF was mixed with the HetR proteins before SDS/PAGE. The proteins were analyzed by SDS/PAGE and transferred onto a polyvinylidene difluoride membrane. The fluorescent bands of DnsF protein adducts (*B1*) were recorded with a digital camera. The DnsF protein adducts were also visualized by Coomassie blue staining (*B2*). Autodegradation of HetR and HetR$_{C48A}$ was observed by incubation of both proteins at 37°C for 1 h before immunoblotting with anti-HetR antibodies (*C*).

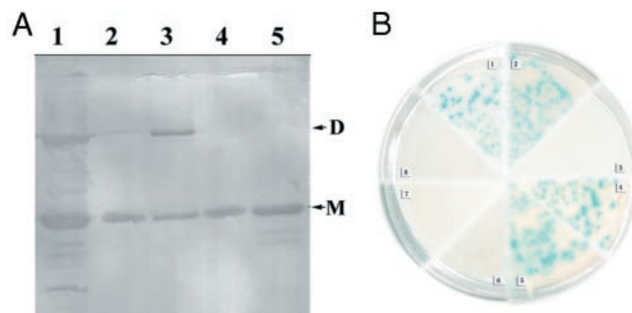

Fig. 2. Formation of HetR homodimer *in vivo*. (*A*) Immunoblotting analysis of HetR proteins from WT211 and C48 of *Anabaena* sp. PCC 7120. The cells were broken in the presence of 1 mM PMSF, and the total cellular extracts were treated with 2% SDS. The proteins were separated by SDS/PAGE followed by electrophoretic transfer onto a polyvinylidene difluoride membrane. HetR proteins were detected with anti-HetR antibodies. Lane 1, rHetR without DTT; lanes 2 and 3, cell extracts from WT211 with and without DTT, respectively; lanes 4 and 5, cell extracts from C48 with and without DTT, respectively. The dimer and monomer are indicated. (*B*) Detection of HetR–HetR interaction by the yeast two-hybrid system. The galactosidase was assayed by the colony-lift method and observed by blue color development. Sections 1 and 2 show the positive controls; section 3 shows the negative control; sections 4 and 5 show HetR–HetR and HetR$_{S179N}$–HetR$_{S179N}$ interactions, respectively; sections 6, 7, and 8 show HetR$_{C48A}$–HetR$_{C48A}$, HetR$_{C48A}$—HetR, and HetR–HetR$_{C48A}$ transformants, respectively.

ing the development of blue color. The plasmids for positive controls were pCL1 and pVA3/pTD1, and pTD1/pLAM5′-1 was used as a negative control according to the manufacturer's instructions.

Electrophoretic mobility-shift assay (EMSA) DNA fragments for EMSA were PCR-amplified and cloned into the pGEM-T-easy vector (Promega). The plasmids containing these fragments were digested with *Eco*RI and the released fragments were isolated and labeled with [α-^{32}P]dATP(111TBq·mmol^{-1}) by using Klenow fragment (Takara). The DNA fragments related to *hetR* were named F$_{hetR}$. F$_{hetR}$-1 and F$_{hetR}$-2 were from the *hetR* upstream region from −318 to −154 and from −891 to −627, respectively; they were amplified with the primers 5′-CAGATAAGTTCCGGATAATAGGG-3′/5′-TATAACTAGCAACAGTGTTGTTA-3′ and 5′-AGAGAATAATTAATAACTCTGGA-3′/5′-TATTATTAAAATTGCATGAATTA-3′, respectively. F$_{hetR}$-3 was a fragment in *hetR* coding the region from +267 to +416, and it was obtained by PCR using the primers 5′-CAAACTGCTCAAGACCTTAGGTT-3′/5′-TTATGCTCAATTTGTCTTTTTTC-3′. Two fragments [F$_{patS}$-1 (−400 to −200) and F$_{patS}$-2 (−199 to +1)] from the promoter region of *patS* were amplified with primers 5′-TAAATCAGTATTTGTTCTCGCAC-3′/5′-ATTAGATTGATATTAAAAATGAC-3′ and 5′-TTTCCGAAAGAGCAGGTATTAAA-3′/5′-TAATCTTAAAATCGGTGAATTAC-3′, respectively. Two fragments [F$_{hepA}$-1 (−521 to −231) and F$_{hepA}$-2 (−230 to +60)] from the promoter region of *hepA* were PCR-amplified with primers 5′-ATGGTGGGCAATGCCCACCATAA-3′/5′- CTCACAATTTTATAGTTGTGCAG-3′ and 5′-CAATACCCACCCTATACTTATTT-3′/5′-TAAATTGTTCTCTTTCCAGAAGC-3′, respectively. The translational start site of *hepA* was according to the published genome sequence (www.kazusa.or.jp/cyano) by Kaneko *et al.* (27) and was 225 bp upstream of the start site reported by Zhu *et al.* (28). EMSA was performed essentially as described by Ausubel *et al.* (29). Briefly, the end-labeled DNA fragments were incubated with HetR, HetR$_{S179N}$, or HetR$_{C48A}$ at a concentration of 1 μM or as indicated in 15 μl of binding buffer [4 mM Tris·HCl (pH 7.5)/12 mM Hepes (pH 7.5)/12% glycerol/50 mM NaCl/10 mM $MgCl_2$] containing 0.1 μg of BSA and 0.5 μg of poly(dI-dC). The binding solutions were incubated at room temperature for 20 min before they were analyzed with a 5% polyacrylamide gel. The radioactive bands were detected by x-ray films (Kodak, Beijing).

Real-Time PCR and SYBR Green I Assays. Real-time PCR was performed with the Prism 7000 system and the reaction SYBR green assay kit (Applied Biosystems). The PCR conditions were as follows: 50°C for 25 min, 95°C for 15 min followed by 40 cycles of 95°C, 55°C, and 72°C for 30 sec each. The relative amount of mRNA (cDNA) was determined by using the real-time PCR product of the calibrated 16S rRNA as a standard.

Other Methods. Overproduction, refolding, and purification of HetR proteins and Dansyl fluoride (DnsF) labeling of HetR were performed as described (22). SDS/PAGE was performed according to Laemmli (30). Immunoblotting for detection of HetR in whole-cell extracts was performed according to Zhou *et al.* (31). Peptide synthesis was performed with Applied Biosystems 433 peptide synthesizer. A Leica fluorescence microscope was used for microscopic studies, and the images were captured by the APOGEE KX cooled charge-coupled device camera (Apogee, Auburn, CA).

Results

Dimerization of HetR Is Required for Heterocyst Formation. When characterizing of the recombinant HetR, we noticed that it could form dimers (22). To investigate the role of HetR dimer formation in regulation of heterocyst differentiation, the residue Cys-48 of the HetR from *Anabaena* sp. PCC 7120 was replaced with an Ala residue through site-specific mutagenesis, and the mutant protein HetR$_{C48A}$ was characterized. Fig. 1 shows that HetR formed dimers whereas HetR$_{C48A}$ was unable to form dimers *in vitro* (Fig. 1*A*) as demonstrated by the lack of the dimer band on SDS/PAGE, suggesting that the HetR dimer was formed through a disulfide bond and the Cys-48 residue was required for the dimerization. DnsF, a specific Ser-type protease inhibitor, could label both the wild-type HetR and HetR$_{C48A}$, and the fluorescent adducts were observed (Fig. 1*B*). Incubation of HetR and HetR$_{C48A}$ at 37°C for 1 h resulted in the same profiles of autodegraded bands (Fig. 1*C*). These results suggest

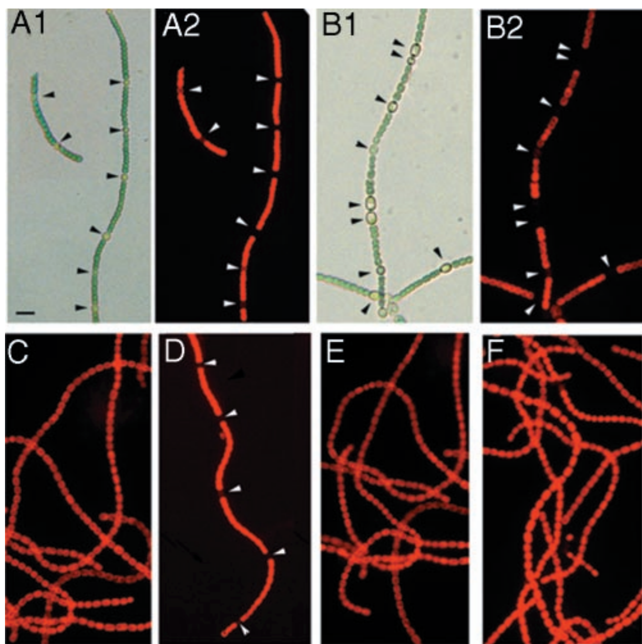

Fig. 3. Bright-field and fluorescence images of the wild-type strain of *Anabaena* sp. PCC 7120 and its derivatives grown with (*C*) or without combined nitrogen. (*A1* and *A2*) Bright-field and fluorescence images of the wild-type filaments, respectively. (*B1* and *B2*) Bright-field and fluorescence images of the wild-type filaments carrying extra copies of *hetR* on pRL25C, respectively. (*C*) Fluorescence image of C48 under nitrogen deprivation conditions for 36 h. (*D*) Fluorescence image of the wild-type filament carrying extra copies of *hetRc48a* on pRL25C. (*E*) Fluorescence image of the wild-type filaments carrying extra copies of *hetRs179n* on pRL25C. (*F*) Fluorescence image of the wild-type filaments with 1 μM PatS pentapeptide (RGSGR) in growth medium. The arrows show the positions of heterocysts. (Scale bar in *A*: 10 μm.)

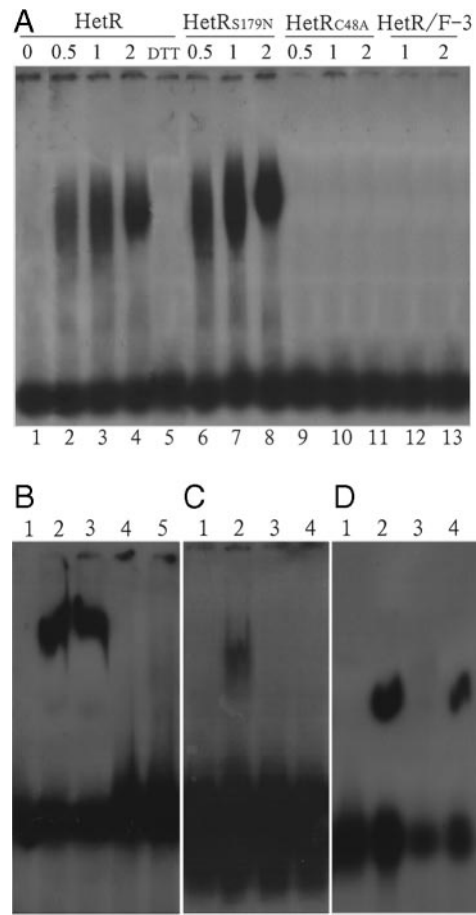

Fig. 4. EMSA for DNA binding by HetR proteins. (*A*) Homodimerization of HetR is required for its DNA-binding activity. A DNA fragment from the *hetR* promoter (F_{hetR}-1, lanes 1–11) and a fragment from the coding region of *hetR* (F_{hetR}-3, lanes 12 and 13) were PCR-amplified and end-labeled with ^{32}P for EMSA. Lanes 1–4, HetR concentration-dependent effect on gel mobility. The concentrations (in μM) are shown above the gels. Lane 5, same as in lane 4 except that 2 mM DTT was included in the reaction solution. Lanes 6–8, concentration-dependent DNA binding by $HetR_{S179N}$. Lanes 9–11, no mobility shift was detected with $HetR_{C48A}$. Lanes 12 and 13, no gel shift was observed at HetR concentrations as shown when F_{hetR}-3 was used for EMSA. (*B*) HetR binding to the fragment F_{hetR}-2 from the *hetR* promoter. Lane 1, no HetR added; lanes 2–4, HetR, $HetR_{S179N}$, and $HetR_{C48A}$ were added, respectively; lane 5, F_{hetR}-3 was used with the addition of HetR. (*C*) HetR binding to the fragment from the *hepA* promoter. Lanes 1 and 2, F_{hepA}-1; lanes 3 and 4, F_{hepA}-2. Lanes 1 and 3, no addition of HetR; lane 2 and 4, with addition of HetR. (*D*) HetR binding to the fragments from the *patS* promoter. Lanes 1 and 2, F_{patS}-1; lanes 3 and 4, F_{patS}-2. Lanes 1 and 3, no addition of HetR; lanes 2 and 4, with addition of HetR. The concentrations of HetR proteins in *B–D* were all 1 μM.

that the mutant protein retained its active Ser site and proteolytic activity.

To investigate whether HetR protein forms dimer *in vivo* and to study the function of the HetR dimer, we introduced the mutant *hetR* gene *hetRc48a* into *hetR*$^{-}$ strain 884a (21) to generate a mutant *Anabaena* strain, C48, confirmed by Southern hybridization (data not shown). Immunoblotting using the antibodies against HetR with the whole-cell extracts (Fig. 2*A*) showed that ≈40% of the total wild-type HetR formed dimer under the growth condition with nitrate. This amount of dimer was comparable to that *in vitro* with the recombinant HetR (Fig. 1*A*). *hetRc48a* was expressed in C48 as demonstrated by the band crossreacting with the anti-HetR antibodies. However, no dimer formation was observed in C48 (Fig. 2*A*). We also used the yeast two-hybrid system to confirm HetR–HetR dimerization (Fig. 2*B*). No interactions were detected between $HetR_{C48A}$ and HetR or between $HetR_{C48A}$–$HetR_{C48A}$, suggesting that HetR forms dimers through a disulfide bond in yeast cells as well. Also shown in Fig. 2*B* is the interaction of $HetR_{S179N}$–$HetR_{S179N}$, a mutant HetR protein that cannot initiate heterocyst differentiation (11).

When C48 was deprived of combined nitrogen, it failed to form heterocysts (Fig. 3*C*), showing that the Cys-48 residue of HetR was required for heterocyst differentiation. When *hetRc48a* was transformed into the wild-type cells on a multiple-copy plasmid pRL25C (32), the resulting strain could form heterocysts and the pattern of heterocysts was normal (Fig. 3*D*), suggesting that $HetR_{C48A}$ did not interfere with the function of HetR. When the wild-type *hetR* gene on pRL25C was introduced into the wild-type cells, multiple contiguous heterocysts were formed (Fig. 3*B*). *hetRs179n* encodes the mutant HetR ($HetR_{S179N}$), and the strain carrying this mutant gene could not differentiate heterocysts (11). When *hetRs179n* on pRL25C was introduced into the wild-type cells, no heterocyst was formed after nitrogen step-down (Fig. 3*E*), suggesting that *hetRs179n* interfered with the normal function of HetR. The synthetic pentapeptide RGSGR, which is present at the C terminus of the PatS peptide, completely blocked heterocyst differentiation at a concentration of 1 μM (Fig. 3*F*), as reported in ref. 16.

HetR Dimerization Is Required for DNA Binding. In a proteomic study of the proteins from *Anabaena* sp. PCC 7120, we detected HetR in a fraction eluted from a heparin column by immunoblotting (data not shown). Because many DNA-binding proteins bind heparin and form dimers, there was a possibility that HetR was a DNA-binding protein. To study whether HetR was capable of

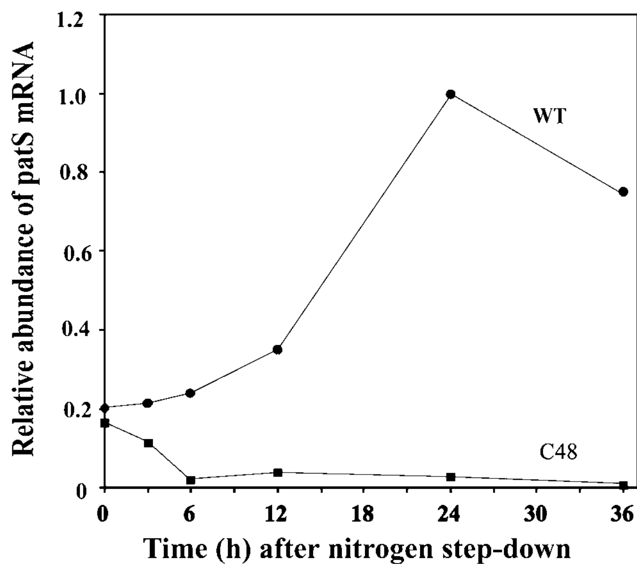

Fig. 5. The expression of *patS* depends on the HetR dimer. Total RNA was isolated from the cells of the wild-type strain (circles) and C48 (squares) at the times indicated after nitrogen step-down and used as templates for reverse transcription. The relative amount of the *patS* mRNA was analyzed by real-time PCR. Each point is an average of four independent values and is normalized against the maximum values of the *patS* mRNA in the wild-type strain at 24 h.

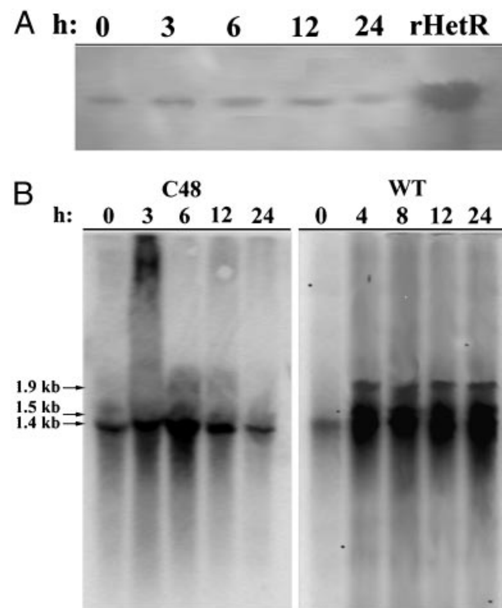

Fig. 6. Analyses of the *hetRc48a* expression in C48 after shifting from a nitrogen replete condition to a nitrogen-deprived condition. (*A*) Immunoblotting analysis of the HetR proteins. Total cellular protein extracts were prepared from cells at the times indicated after nitrogen step-down and separated by SDS/PAGE before electrophoretic transfer to a polyvinylidene difluoride membrane. HetR was detected as described in Fig. 2. (*B*) Northern blotting analysis of the expression of *hetR* and *hetRc48a*. Total RNA was isolated from the cells at the times indicated after nitrogen deprivation and separated with 1.5% agarose gel electrophoresis before transfer to a nylon membrane. The mRNA of *hetR* and *hetRc48a* was detected by using a ^{32}P-labeled *hetR* probe. The transcript sizes are shown on the left. The smear on top of the 3-h lane in C48 could probably be resulted from unremoved polysaccharides in the RNA sample.

binding DNA, we selected a DNA fragment from the *hetR* promoter region for the DNA-binding study because *hetR* is autoregulatory (21). The fragment of −318 to −154 from the *hetR* promoter (F_{hetR}-1) and a fragment in *hetR* coding region (+267 to +416, F_{hetR}-3) were selected for EMSA (Fig. 4*A*). We found that HetR could bind the F_{hetR}-1 in a protein concentration-dependent fashion (Fig. 4*A*, lanes 1–4). No mobility shift was observed with the F_{hetR}-3 (Fig. 4*A*, lanes 12 and 13), indicating that the DNA binding by HetR was specific. $HetR_{C48A}$ failed to bind F_{hetR}-1 at the protein concentrations we tested (Fig. 4*A*, lanes 9–11). Like HetR, $HetR_{S179N}$ could bind F_{hetR}-1 in a protein concentration-dependent fashion (Fig. 4*A*, lanes 6–8). We systematically tested the promoter region of *hetR* from *Anabaena* sp. PCC 7120 and found that HetR bound another fragment, the F_{hetR}-2 (−891 to −627, Fig. 4*B*). $HetR_{C48A}$ did not bind to this fragment, whereas $HetR_{S179N}$ did, as in case of F_{hetR}-1 (Fig. 4*B*).

The expression of *hepA*, which is involved in heterocyst envelope synthesis, depends on the expression of *hetR* (21). *patS* encodes a strong inhibitor of heterocyst differentiation, and its up-regulation is located in heterocysts and proheterocysts (16). We chose the promoters of these two genes for DNA-binding studies. Two fragments of the *hepA* promoter [F_{hepA}-1 (−521 to −231) and F_{hepA}-2 (−230 to +60)] and two fragments of the *patS* promoter [F_{patS}-1 (−400 to −200) and F_{patS}-2 (−199 to +1)] were used for EMSA. HetR bound F_{hepA}-1 (Fig. 4*C*), F_{patS}-1, and F_{patS}-2 (Fig. 4*D*) and did not bind F_{hepA}-2 (Fig. 4*C*), suggesting again that the DNA binding by HetR was specific. $HetR_{C48A}$ could not bind any of the fragments used in these studies (data not shown).

HetR DNA Binding Is Required for the Up-Regulation of *patS* and *hetR*. The fact that HetR bound to the *patS* promoter suggested that the up-regulation of *patS* depended on HetR. We used real-time PCR to investigate whether the HetR dimer was required for the up-regulation of *patS*. The *patS* mRNA abundance in the wild-type strain and C48 after nitrogen step-down was determined (Fig. 5). In the wild-type cells, the *patS* mRNA level did not increase until 6 h after nitrogen deprivation. The peak level (5-fold increase) was reached 24 h after nitrogen step-down. In C48, no up-regulation of the *patS* expression could be detected during the entire process of heterocyst differentiation, suggesting that the expression of *patS* depended on the HetR dimer.

The up-regulation of *hetR* is one of the earliest events during heterocyst differentiation (11, 19, 21), and the increased expression of *hetR* is maintained during the process of differentiation (31). When C48 was subjected to nitrogen step-down, the amount of $HetR_{C48A}$ did not show an apparent increase, and the amount of $HetR_{C48A}$ 24 h after nitrogen step-down was only 60% of the initial amount (Fig. 6*A*). Northern hybridization (Fig. 6*B*) showed an initial increase of the *hetRc48a* transcript 3 h after the shift. The transcript level reached its peak at 6 h and then declined within 6 h. At 24 h after the shift, the level of the *hetR* transcript was only 70% of the initial level. In the wild-type cells, the induction of *hetR* was fast and the high level of the expression was maintained throughout the process of differentiation (24 h). Another critical difference between the wild-type strain and C48 shown in Fig. 6*B* is that only one major transcript size (1.4 kb) was detected in the mutant strain whereas a 1.5-kb transcript was much reduced and the 1.9-kb transcript observed in the wild-type strain (19) was largely missing. The abundance of *hetR* mRNA was also determined by real-time PCR (Fig. 7). The *hetRc48a* transcript in C48 increased initially and reached to the peak level in 6 h; it declined rapidly to the original level within an additional 6 h.

PatS Pentapeptide Inhibits the DNA Binding of HetR. The strong inhibition of heterocyst formation by PatS pentapeptide (Fig. 3

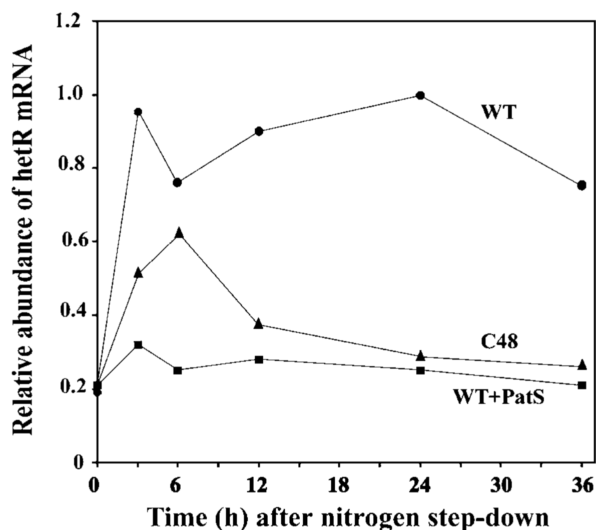

Fig. 7. Real-time PCR analysis of the expression of *hetR*. The wild-type cells were subjected to a nitrogen step-down with (squares) or without (circles) 1 μM PatS pentapeptide, and total RNA was isolated at the times indicated for real-time PCR. The expression of *hetRc48a* in C48 after nitrogen step-down is also shown (triangles). Each point is an average of four independent values, and all points are normalized against the maximum value of the *hetR* mRNA at 24 h after nitrogen step-down.

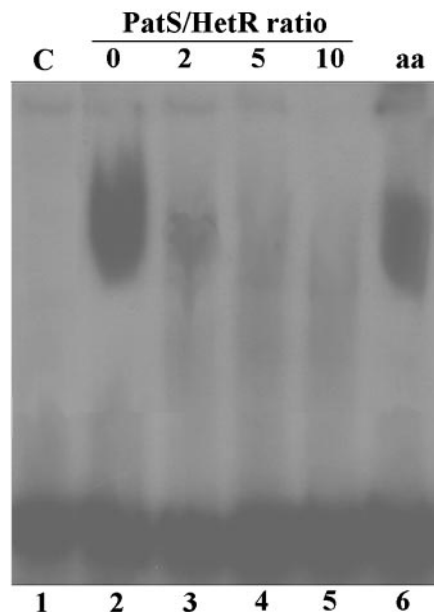

Fig. 8. The PatS pentapeptide inhibits the DNA-binding activity of HetR. EMSA with F_{hetR}-1 was performed as described in Fig. 4. The PatS pentapeptide RGSGR (lanes 2–5) or a mixture of the 3 aa present in the pentapeptide (lane 6) in a same molar ratio as in lane 5 was added to the binding solutions before electrophoresis. The HetR concentration was 1 μM, and the molar ratios of PatS/HetR are shown on top of the gel. Lane 1, no HetR was added to the reaction solution.

and ref. 16) was an indication that it inhibited the early process of differentiation, and HetR could be its target. This finding led us study the effect of PatS pentapeptide on *hetR* up-regulation. Fig. 7 shows that when the PatS pentapeptide was added to the growth medium at a final concentration of 1 μM, it blocked *hetR* induction completely after nitrogen step-down.

Because *hetR* induction depended on HetR dimer required for DNA binding, the results shown in Fig. 7 led us to investigate whether the pentapeptide inhibited the DNA-binding activity of HetR (Fig. 8). When the molar ratio of PatS/HetR in the binding solution was 5, the pentapeptide nearly completely abolished the HetR-dependent gel mobility shift. Partial inhibition of the shift could be observed when the ratio was 2. When the equivalent molar ratio was 10, the mixture of the amino acid present in the pentapeptide had no effect on HetR-induced gel shifting. We found no evidence that the PatS pentapeptide had any effect on either the formation of the HetR dimer and or HetR autodegradation (data not shown).

Discussion

The experimental results shown in this study provide evidence that HetR is a DNA-binding protein and that this activity is required for heterocyst differentiation. Although the HetR protein does not contain any apparent DNA-binding motifs by its primary sequence, the results in Fig. 4 clearly show that HetR binds DNA. HetR binds the promoters of *hepA*, *patS*, and *hetR* itself and is required for the up-regulation of these genes (Figs. 5 and 6). The requirement for HetR to bind DNA could be the reason that the amount of HetR controls heterocyst frequency (19) because HetR binds DNA in a concentration-dependent fashion (Fig. 4).

The DNA binding of HetR requires its dimerization because $HetR_{C48A}$, which could not form homodimers through disulfide bonds either *in vitro* (Fig. 1) or *in vivo* (Fig. 2), has no DNA-binding activity (Fig. 4). The lack of dimerization of $HetR_{C48A}$ is probably the only reason that it did not bind DNA, because it could be labeled by DnsF and had autodegradation activity (Fig. 1), strongly suggesting that the protein was correctly folded. Protein dimerization is often required by DNA-binding proteins. For example, the AraC transcriptional activators that are involved in sugar metabolism require dimerization (33). In cyanobacteria, NtcA is a key regulator of nitrogen metabolism, and it influences the early process of heterocyst differentiation (9, 34, 35). Existing evidence suggests that NtcA functions as a dimer (36).

HetR dimerization is required for heterocyst formation because C48 failed to form heterocysts (Fig. 3) even though *hetRc48A* was expressed (Fig. 2). *hetRc48a* behaves differently from *hetR*. Contrary to HetR in the wild-type cells (23, 31), no apparent increase of $HetR_{C48A}$ could be observed when C48 was subjected to nitrogen step-down. The results of Northern blotting and real-time PCR showed an initial increase followed by a decline of the *hetRc48a* mRNA after nitrogen step-down (Figs. 6*B* and 7). The *hetR* transcript pattern also changed in C48. The 1.9-kb transcript of *hetR* observed in the wild-type cells after nitrogen step-down was not detected in C48, similar to that reported for strain 216 (19). We conclude that C48 fails to form heterocyst because *hetRc48a* lacks autoregulation due to the inability of $HetR_{C48A}$ to bind DNA. For this reason, overexpression of *hetRc48a* did not interfere with the normal function of *hetR*, whereas overexpression of *hetRs179n* inhibited heterocyst formation (Fig. 3). It is likely that the $HetR_{S179N}$ dimer overproduced in the wild-type cells outcompeted HetR for the DNA-binding sites and thus interfered with heterocyst formation (Fig. 3).

Previous reports (22, 23) and the results presented here suggest that both the protease activity and the DNA-binding activity of HetR are required for its functions. The fact that strain 216 carrying *hetRs179n* is unable to differentiate heterocyst whereas $HetR_{S179N}$ binds to DNA shows the importance of HetR protease activity in the regulation of heterocyst formation. Another strain carrying a mutant *hetR* gene encoding $HetR_{S152A}$ also fails to form heterocyst (23). Proteases play important roles in cell differentiation and development (37). The protease Lon, which has several important regulatory functions in both bacte-

rial and eukaryotic cells, is a DNA-binding protein (38). In *Pseudomonas syringae*, Lon regulates type III protein secretion by degrading an alternative σ factor (39). It is possible that HetR binds DNA and digests a repressor to initiate the up-regulation of the genes involved in heterocyst differentiation. Another possibility is that HetR is a transcriptional activator as previously postulated (19). So far, we have not been able to observe interactions between HetR and any components of the RNA polymerase from *Anabaena* sp. PCC 7120 with the two-hybrid system (data not shown). Further study is needed to know whether HetR functions as a transcriptional activator.

The results shown in Fig. 8 provide evidence that PatS regulates the heterocyst pattern by inhibiting the DNA-binding activity of HetR. The mechanism of the inhibition is not known. The PatS pentapeptide RGSGR has a symmetric structure with two basic arginine residues; it could occupy the DNA-binding site of HetR and prevent its DNA binding.

Two features about *hetR* and *patS* might be important in initiating differentiation and maintaining the pattern of heterocysts. First, the timing of the up-regulation of *hetR* and *patS* is critical. The up-regulation of *patS* depends on HetR and is several hours behind that of *hetR* (Figs. 5 and 7). This would result in a low PatS/HetR ratio in proheterocysts and ensure that PatS does not inhibit HetR in these cells. Second, the autodegradation activity of HetR might be important in keeping a high ratio of PatS/HetR in vegetative cells for a full inhibition of DNA binding by HetR. HetR could be modified upon nitrogen deprivation (31), and *patA* might be involved in this process (19, 40). The modification of HetR could play an important role in the regulation of HetR autodegradation and PatS/HetR interaction.

Our results show that cell–cell communications through molecular interactions between HetR and PatS play a key role in controlling the heterocyst pattern. The functions of HetR and PatS and the relationship of these two genes agree well with the model proposed for biological pattern formation by Turing (41) and by Meinhardt and Gierer (42). The model predicts coexistence of a differentiation activator (such as HetR) and a diffusible differentiation suppressor (such as PatS) in a differentiating cell, and the expression of the suppressor depends on the activator.

The DNA sequence that HetR binds could not be revealed by comparison of the DNA fragments used in our study. Determining the DNA sequence that HetR binds should help to reveal how many genes are regulated by HetR.

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