

NrrA Directly Regulates Expression of *hetR* during Heterocyst Differentiation in the Cyanobacterium *Anabaena* sp. Strain PCC 7120[∇]

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Heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120 requires NtcA, the global nitrogen regulator in cyanobacteria, and HetR, the master regulator of heterocyst differentiation. Expression of *hetR* is upregulated by nitrogen deprivation, and its upregulation depends on NtcA. However, it has not yet been revealed how NtcA regulates the expression of *hetR*. In the experiments presented here, it was confirmed that NrrA (All4312), a nitrogen-responsive response regulator, was required for the upregulation of *hetR*. The use of the nitrogen-responsive transcription initiation sites (TISs) for the *hetR* gene depended upon NrrA. NrrA bound specifically to the region upstream of TISs located at positions –728 and –696 in vitro. Overexpression of *nrrA* resulted in enhanced *hetR* expression and heterocyst formation. A molecular regulatory cascade is proposed whereby NtcA upregulates the expression of *nrrA* upon limitation of combined nitrogen in the medium and then NrrA upregulates the expression of *hetR*, leading to heterocyst differentiation.

Heterocysts are terminally differentiated cells of cyanobacteria that are capable of fixing atmospheric nitrogen. Upon limitation of combined nitrogen in the medium, particular vegetative cells change their form into heterocysts with a regular spacing of 10 to 15 cells (10, 12, 29). In the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120, about 10% of genes on the chromosome are estimated to be upregulated during heterocyst development (6, 7). Heterocysts markedly differ from vegetative cells in physiological and morphological aspects (30). Heterocyst development provides an excellent model for studying cell differentiation and multicellular pattern formation.

The *hetR* gene is a master gene that regulates heterocyst differentiation. While mutation in the *hetR* gene blocks early steps in differentiation, extra copies of *hetR* result in the formation of multiple contiguous heterocysts (Mch phenotype) under nitrogen-depleted conditions (4). Expression of *hetR* from the *petE* promoter, which is regulated by copper, induces heterocyst differentiation irrespective of cellular nitrogen status (5). The level of the *hetR* transcripts increases shortly after nitrogen deprivation, and its expression is localized to heterocysts (2). The upregulation of *hetR* depends on *ntcA* (9).

The *ntcA* gene, which is required for heterocyst development (9, 28), encodes a transcriptional regulator that globally controls nitrogen metabolism in cyanobacteria (19, 27). The NtcA protein, belonging to the cyclic AMP receptor protein family of bacterial regulators, binds to promoter regions containing the sequence signature GTAN₈TAC (12), and an eight-base pal-

indromic sequence, TGTAN₈TACA, is shown to be the optimal NtcA-binding sequence by in vitro selection (15). The DNA-binding activity of NtcA is enhanced by 2-oxoglutarate (2-OG) (17, 26), and NtcA-dependent transcription from the *glnA* promoter of *Synechococcus* sp. strain PCC 7942 increases in the presence of 2-OG in vitro (25). 2-OG is proposed to be a signaling molecule that transmits information regarding cellular nitrogen status (24) and that triggers heterocyst differentiation (17). The NtcA-dependent promoters of *hetR* do not contain the NtcA recognition sequence, and NtcA does not bind to DNA fragments corresponding to the *hetR* promoter regions (23). Thus, the molecular basis of the NtcA-dependent regulation of *hetR* remains unknown.

Recently we found that the *nrrA* gene (all4312), encoding a response regulator of the OmpR family, was involved in the regulation of heterocyst development (6). The *nrrA* gene is upregulated by nitrogen deprivation, but the induction of *nrrA* does not take place in the *ntcA* mutant strain (6, 22). The promoter sequence of the *nrrA* gene is consistent with the NtcA-activated promoter sequence, and NtcA binds to the promoter region of *nrrA* (22). The deletion of *nrrA* delays heterocyst development and diminished *hetR* induction under nitrogen deprivation (6).

In this study, we investigated the regulation of *hetR* expression by NrrA. The NrrA protein showed sequence-specific binding to the promoter region of *hetR*. In *Anabaena* sp. strain PCC 7120 having extra copies of the *nrrA* gene on a plasmid, the amount of the *hetR* transcript increased and heterocyst formation was enhanced. These results indicate that NrrA directly regulates *hetR* expression. A molecular cascade between activation of NtcA and upregulation of *hetR* in the early stage of heterocyst differentiation is linked by NrrA, i.e., NtcA upregulates the expression of *nrrA*, and then NrrA upregulates the expression of *hetR* during heterocyst differentiation.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *Anabaena* sp. strain PCC 7120 and its derivatives were grown in BG-11 medium as described previously (6). The *Escherichia coli* strains used as hosts were JM109 [*recA1 endA1 gyrA96 thi hsdR17*(r_K⁻m_K⁺) *supE44 relA1 Δ(lac-proAB)/F'* (*traD36 proAB lacI^q lacZΔM15*)] for cloning and BL21(DE3) [*F*⁻ *ompT hsdS*(r_B⁻m_B⁻) *dcm gal λ*(DE3)] for the expression of recombinant proteins. *E. coli* strains were grown in Luria-Bertani medium (21).

Plasmid constructions. A DNA fragment containing the *nrrA* promoter and coding regions was amplified by PCR using the primer pair 4312F2 (5'-CAGG CTGAAATGTGAAGGGTA-3') and 4312R (5'-AACCAAGCCGATGAAGA ATG-3') and was cloned into the EcoRV site of pBluescript II SK+ (Stratagene, CA). This fragment was excised by digestion with BamHI and KpnI and was cloned between the BamHI and KpnI sites of pRA201 (8) to construct pRA4312.

For construction of an expression plasmid for the histidine-tagged NrrA (His-NrrA) protein, a DNA fragment containing the *nrrA* gene was amplified by PCR using the primer pair NrrA-1F (5'-GACATATGGGTTTCGGTTTGTATTGAA-3') and NrrA-R (5'-ATGTCGACTCCCTCTGCCTCTACTAC-3'). The amplified DNA fragment was cloned between the NdeI and SalI sites of the pET-28a expression vector (Merck KGaA, Darmstadt, Germany). The resulting plasmid, pENrrA1, contains the *nrrA* gene fused to the His tag sequence.

RNA gel blot analysis and primer extension analysis. Extraction of total RNA and analysis of RNA gel blots were carried out as described previously (6).

Two fluorescence-labeled primers, IRD-hetR-120 (5'-GGTCAAGATGCTC ATTCTC-3') and IRD-hetR-603 (5'-TGCTCTTATGGCAGTGTAGG-3'), were used for primer extension analysis as described previously (6). DNA sequencing was carried out with a SequiTherm EXCELII DNA sequencing kit LC (EPICENTRE, WI) using a plasmid pBhetRu containing the region from -967 to +25 with respect to the translation start site of the *hetR* gene as a template. The cDNA and sequencing products were analyzed with a model 4200 DNA sequencer (LI-COR Biosciences, NE).

Expression and purification of His-NrrA. *E. coli* BL21(DE3) cells harboring pENrrA1 were grown at 37°C in 1 liter of Luria-Bertani medium supplemented with kanamycin (30 μg/ml). The recombinant gene was expressed in exponentially growing cells (optical density at 600 nm of 0.6) by adding 1 mM isopropyl-β-D-thiogalactopyranoside. After 1 h of incubation, the cells were harvested by centrifugation, washed with buffer A (20 mM Tris-HCl [pH 8.0], 0.3 M NaCl, 10% glycerol), resuspended in 30 ml of buffer A, and disrupted with a French pressure cell (20,000 lb/in²). The cell extract was centrifuged at 16,000 × g for 10 min, and the supernatant was further centrifuged at 150,000 × g for 60 min. The 150,000 × g supernatant was loaded onto a HiTrap chelating HP column (GE Healthcare Bio-Science, NJ) equilibrated with buffer A, and the proteins were eluted using a gradient of 30, 60, 100, and 250 mM imidazole in buffer A. The 250 mM imidazole fraction containing purified His-NrrA was loaded onto a PD-10 column (GE Healthcare Bio-Science) equilibrated with buffer B (20 mM Tris-HCl [pH 8.0], 0.1 M NaCl, 10% glycerol), and the protein was eluted with buffer B.

Gel mobility shift assay. Two DNA fragments, F1 and F2, were generated by PCR using the primer pair hetRF-876 (5'-AACTCTGGACTTCTGGCTCA-3') and hetRR-659 (5'-GACAGGTAATTCCAACCTTAGCAA-3') and primer pair hetRF-440 (5'-CACCTCAAGGAGAGATTGTGC-3') and hetRR-182 (5'-CG TGCTGTATCTTACCGAAA-3'), respectively. The digoxigenin (DIG)-labeled primers, hetRF-876 and hetRF-440, were purchased from Japan Bio Services (Saitama, Japan). The DIG-labeled DNA fragments (0.1 ng) were incubated with His-NrrA in 20 μl of the binding buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol, 0.5 μg/ml of poly(dI-dC)] for 30 min at room temperature. The mixtures were subjected to electrophoresis on a native 5% polyacrylamide gel and transferred to a nylon membrane (Hybond-N+; GE Healthcare Bio-Science). The probes were detected with an alkaline phosphatase-conjugated anti-DIG Fab fragment and a chemiluminescence reagent CDP-Star (Roche Diagnostics, Basel, Switzerland) according to the recommendation of the supplier.

DNase I footprinting analysis. A fluorescence-labeled DNA fragment was generated by PCR using the primer pair hetRF-876 and IRD-hetR-603. The fluorescence-labeled DNA fragment (500 ng) was incubated with His-NrrA in 25 μl of the binding buffer containing 40 μg/ml of poly(dI-dC) for 30 min at room temperature. Then, 0.2 U of DNase I (RNase-free) (TaKaRa Bio, Shiga, Japan) was added to the reaction mixture, which was then incubated for 10 seconds at 30°C. The reaction was terminated by the addition of 25 μl of 20 mM EDTA. The reaction mixture was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1), and DNA was collected by ethanol precipitation. The partially digested

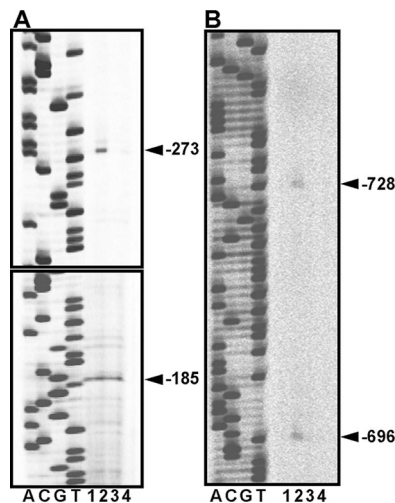


FIG. 1. Primer extension analyses of the expression of *hetR* in *Anabaena* sp. strain PCC 7120 and the *nrrA* deletion mutant DR4312S. RNA was isolated from *Anabaena* sp. strain PCC 7120 (lanes 1 and 2) and DR4312S (lanes 3 and 4) cells grown with nitrate (lanes 1 and 3) or cells subjected to nitrogen deficiency for 3 h (lanes 2 and 4). Oligonucleotides used as primers were IRD-hetR-120 (A) and IRD-hetR-603 (B). Sequencing ladders were generated with the corresponding primers and pBhetRu. The arrowheads indicate the positions of the putative transcription initiation sites.

DNA fragments were analyzed with a model 4200 DNA sequencer (LI-COR Biosciences).

Other analytical procedures. Protein concentration was determined by the method of Bradford (3) using bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in polyacrylamide gels containing 0.1% SDS by the method of Laemmli (16).

RESULTS

NrrA upregulates the *hetR* gene under nitrogen deprivation.

Figure 1 shows the result of primer extension analyses of the *hetR* transcripts using RNA isolated from *Anabaena* sp. strain PCC 7120 and the *nrrA* deletion mutant strain DR4312S (6). In *Anabaena* sp. strain PCC 7120, four putative transcription initiation sites (TISs) were detected as reported previously (5, 23). Under the present experimental conditions, the TISs reported at positions -271 and -184 were detected at positions -273 and -185, respectively. The use of the TIS located at -185 was independent of nitrogen status, while the use of the other three TISs (-728, -696 and -273) was activated by nitrogen deprivation (Fig. 1, lanes 1 and 2). Deletion of *nrrA* blocked induction of transcription from the nitrogen-responsive TISs after 3 h of nitrogen deprivation (lanes 3 and 4), indicating that the use of these TISs depended on NrrA. The constitutive expression from position -185 was not affected by deletion of *nrrA*, suggesting that NrrA was not required for transcription from this TIS.

NrrA binds to the region upstream of the *hetR* gene. The NrrA protein was expressed as a recombinant protein fused to the His tag sequence at the amino terminus (His-NrrA) in *E. coli* cells bearing plasmid pENrrA1 (Fig. 2A). Two bands of affinity-purified His-NrrA were observed by SDS-PAGE (Fig. 2A, lane 2) and both of them had the His tag sequence (lane 4). The molecular mass of a major band was estimated to be 30

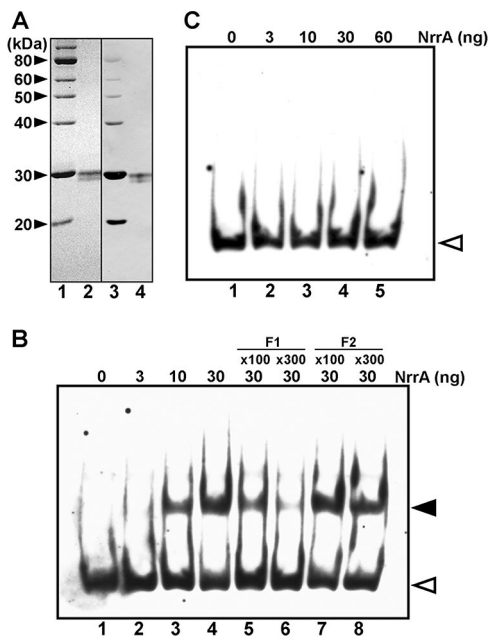


FIG. 2. (A) Purification of His-tagged NrrA. SDS-PAGE was carried out using a 12% polyacrylamide gel. Lanes 1 and 3, His-tagged molecular size markers; lanes 2 and 4, His-tagged NrrA (1 μ g). The gel was stained with Coomassie brilliant blue (lanes 1 and 2). His-tagged proteins were detected with horseradish peroxidase-conjugated N-nitrotriacetic acid (lanes 3 and 4). (B and C) Gel mobility shift assays with His-NrrA and the regions upstream of the *hetR* gene. The binding of His-NrrA to fragment F1 (B) and fragment F2 (C), corresponding to the region from -876 to -659 and from -440 to -182 with respect to the translation start site of the *hetR* gene, respectively, was examined. His-NrrA was added in the amounts indicated above each lane. In panel B, nonlabeled F1 (lanes 5 and 6) and F2 (lanes 7 and 8) fragments were added at the indicated excess amounts. Open arrowheads indicate the positions of the free probe, and a closed arrowhead indicates the position of the NrrA-F1 complex.

kDa, corresponding closely to the theoretical value. The minor band observed just below the major band had probably been cleaved at the carboxyl terminus.

Gel mobility shift assays were carried out with purified His-NrrA and DNA fragments of the region upstream of *hetR* (Fig. 2B and C). His-NrrA reduced the electrophoretic mobility of the DNA probe F1 that included the TISs located at positions -728 and -696 , and the amount of the NrrA-F1 complex increased in proportion to the concentration of His-NrrA (Fig. 2B, lanes 1 to 4). However, His-NrrA did not form a complex with probe F2 that included the TISs at positions -273 and -185 (Fig. 2C). The band intensity of the NrrA-F1 complex was reduced by the addition of a nonlabeled F1 fragment but was not affected by the addition of a nonlabeled F2 fragment (Fig. 2B, lanes 5 to 8). These results show that His-NrrA binds to the probe F1, corresponding to the region from -876 to -659 with respect to the translation start site of the *hetR* gene, in a sequence-specific manner.

Figure 3 shows the result of a DNase I footprinting assay performed using His-NrrA and the region upstream of *hetR*. The His-NrrA protein protected a region of 27 bp, covering -844 to -818 with respect to the translation start site of the *hetR* gene, from DNase I digestion. The region protected by NrrA

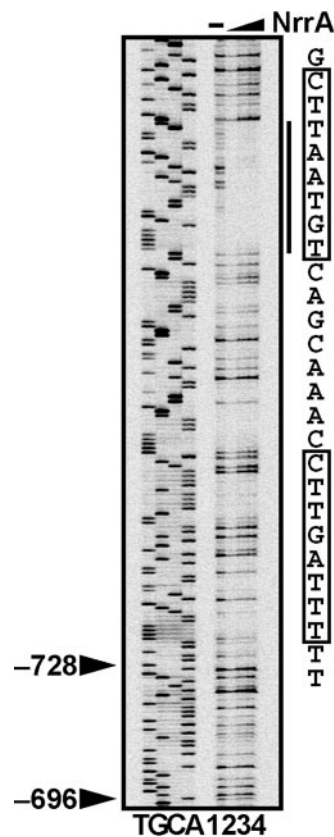


FIG. 3. DNase I footprinting assay with His-NrrA and the region upstream of the *hetR* gene. The patterns of fragments resulting from digestion with DNase I of the fluorescence-labeled DNA fragment containing the region from -876 to -603 with respect to the translation start site of the *hetR* gene are shown. His-NrrA was added in the concentration of 0 (lane 1), 0.25 (lane 2), 0.5 (lane 3), and 1 μ M (lane 4) in each reaction. Sequencing ladders were generated with primer IRD-*hetR*-603 and pBhetRu. Arrowheads indicate the positions of the transcription initiation sites. A region protected from DNase I digestion is shown by a solid line, and its sequence is shown on the right. Candidates for the NrrA recognition sequence are indicated by boxes.

includes tandemly repeated sequences CTT(A/G)AT(G/T)T. The NrrA protein belongs to the OmpR family, and response regulators of the OmpR family recognize tandemly repeated sequences (13, 20).

Extra copies of the *nrrA* gene enhance the expression of *hetR*. A plasmid carrying the *nrrA* gene, pRA4312, and the control plasmid pRA201 were transferred into *Anabaena* sp. strain PCC 7120 by conjugation, and transformants were selected for resistance to neomycin. In *Anabaena* sp. strain PCC 7120 bearing pRA201, the *nrrA* transcript was not detected in the presence of nitrate and was induced within 3 h after nitrogen deprivation (Fig. 4A). In the strain bearing pRA4312, the *nrrA* transcript was detected even in the presence of nitrate and markedly increased after nitrogen deprivation (Fig. 4A). Overexpression of the *nrrA* gene increased the transcript level of *hetR*, while the increase was less than that of the *nrrA* transcript (Fig. 4B). The amount of the *hetR* transcripts increased by twofold after 3 and 8 h of nitrogen deprivation and by threefold after 24 h of nitrogen deprivation in the strain bearing pRA4312.

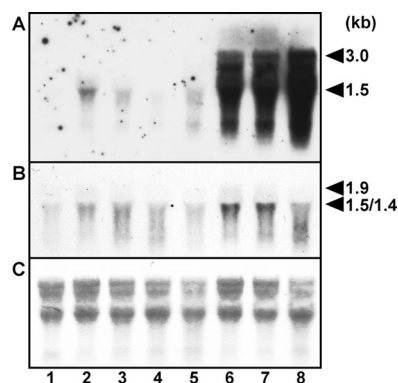


FIG. 4. RNA gel blot analysis in *Anabaena* sp. strain PCC 7120 having extra copies of the *nrrA* gene on a plasmid. Total RNA was isolated from nitrate-grown cells (lanes 1 and 5) and cells subjected to nitrogen deficiency for 3 h (lanes 2 and 6), 8 h (lanes 3 and 7), and 24 h (lanes 4 and 8). Lanes 1 to 4, *Anabaena* sp. strain PCC 7120 bearing the control plasmid pRA201; lanes 5 to 8, *Anabaena* sp. strain PCC 7120 bearing pRA4312, a plasmid carrying the *nrrA* gene. The filters were hybridized to an *nrrA* probe (A) and a *hetR* probe (B). RNA was stained with methylene blue (C). The sizes of transcripts are shown to the right of the panels.

Figure 5 shows the effect of overexpression of the *nrrA* gene on heterocyst formation. At least 1,000 cells each from two independently grown cultures were scored to determine the frequency of heterocysts. The heterocyst frequency in *Anabaena* sp. strain PCC 7120 bearing pRA201 grown with nitrate was 0.8%, while it was 2.1% in the strain bearing pRA4312 (Fig. 5A and B). After nitrogen deprivation, heterocyst differentiation was induced and a multiple contiguous heterocyst (Mch) phenotype with pairs of heterocysts and occasionally groups of three or four heterocysts was observed in the multicopy *nrrA* strain (Fig. 5C). Many free heterocysts detached from filaments, probably resulting from breakage of labile connections between contiguous heterocysts, were also observed (Fig. 5C). Heterocyst formation was completely repressed by the addition of ammonium in both strains (data not shown).

DISCUSSION

NtcA and HetR play the main regulatory roles in the early stage of heterocyst development. The induction of *hetR* expression during heterocyst differentiation requires NtcA, whereas molecular mechanisms of regulation of the *hetR* expression by NtcA have not been revealed (9, 23). In this study, we showed that the *nrrA* gene was required for the upregulation of *hetR* by nitrogen deprivation (Fig. 1) and that NrrA specifically bound to the region upstream of *hetR* in vitro (Fig. 2 and 3). In *Anabaena* sp. strain PCC 7120 having extra copies of the *nrrA* gene, the amount of *hetR* transcripts and the frequency of heterocysts were increased (Fig. 4 and 5). Excessive NrrA could increase the level of the expression of *hetR* in vivo and lead to supernumerary heterocysts. These results indicate that NrrA directly regulates the expression of *hetR* during heterocyst differentiation.

Figure 6 shows a model for sequential activation of gene expression in the early stage of heterocyst differentiation. Lim-

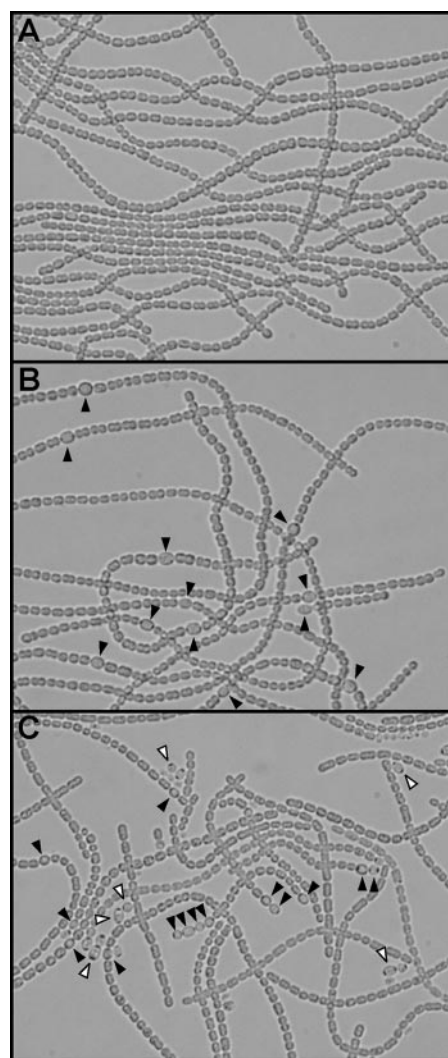


FIG. 5. Light microscopy of *Anabaena* sp. strain PCC 7120 having extra copies of the *nrrA* gene. *Anabaena* sp. strain PCC 7120 bearing pRA201 was grown with nitrate (A). *Anabaena* sp. strain PCC 7120 bearing pRA4312 was grown with nitrate (B) or without combined nitrogen (C). Closed arrowheads show heterocysts. Open arrowheads show heterocysts detached from filaments.

itation of combined nitrogen in the medium causes accumulation of 2-oxoglutarate within the cells of *Anabaena* sp. strain PCC 7120 (17, 18). It has been proposed that accumulated 2-OG serves as the nitrogen limitation signal triggering the signal pathways that lead to heterocyst differentiation (17, 18). 2-OG enhances the DNA-binding activity of NtcA (17). NtcA directly regulates the expression of genes involved in heterocyst maturation, such as *hetC* and *devBCA*, as well as genes involved in nitrogen assimilation, such as *glnA* and the *nir* operon (11). NtcA also regulates the expression of *nrrA* (22). The *nrrA* gene is upregulated by nitrogen deprivation and is involved in regulation of heterocyst differentiation. In the *nrrA* deletion mutant, heterocyst formation after nitrogen deprivation is delayed, and the *hetR* induction by nitrogen deprivation is reduced up to 24 h (6). It was shown that the *nrrA* gene was required for the initiation of transcription from the nitrogen-

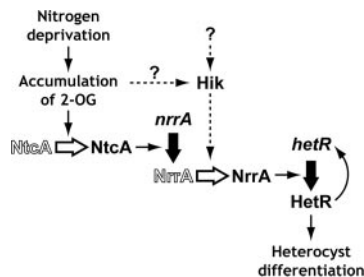


FIG. 6. A model for a regulatory cascade in the early stage of heterocyst differentiation in *Anabaena* sp. strain PCC 7120. Thick black arrows represent upregulation of gene expression. Open arrows represent activation of proteins. A hitherto unidentified pathway that regulates the NrrA activity is indicated by broken arrows. See the text for details. Hik, histidine kinase.

responsive TISs of *hetR* located at positions -728 , -696 , and -273 (Fig. 1). The 1.9-kb transcript of *hetR* originates from positions -728 and -696 , and the 1.5-kb transcript originates from position -273 (5). In the *nrrA* mutant, the 1.9-kb transcript is not detected, and the level of induction of the 1.5-kb transcript is reduced after nitrogen deprivation (6). NrrA bound to the region upstream of the TISs at positions -728 and -696 (Fig. 2B and 3), suggesting that transcription from these TISs is directly regulated by NrrA. Use of the TIS at position -728 also depends on NtcA (23). Thus, the NtcA-dependent regulation of *hetR* must be mediated by NrrA. HetR is a DNA-binding protein and binds to the region around the TISs at positions -728 and -696 (14). However, HetR is not required for transcription from these TISs (23). Functions of HetR in transcription from these TISs remain unknown. Expression of *hetR* is subjected to autoregulation, a key aspect of the regulation of heterocyst differentiation (2). Transcription from the TIS at position -273 requires HetR itself (23), and HetR binds to the DNA fragment around this TIS (14). NrrA was also required for transcription from this TIS (Fig. 1 and 2C). In the *nrrA* deletion mutant, absence of the induction of *hetR* from the TISs at positions -728 and -696 would diminish the autoregulatory induction from the TIS at position -273 . It has been reported that the *hetF* gene is involved in the regulation of *hetR* in *Nostoc punctiforme* (31). The *hetF* gene is required for the quick induction of *hetR* after nitrogen deprivation and localization of the HetR protein in heterocysts. The HetF protein is suggested to be a protease (1), but its role in a regulatory cascade of heterocyst differentiation has not been revealed yet.

NrrA is a response regulator belonging to the OmpR family, and thus, its activity is regulated by a sensory histidine kinase. An aspartate residue that corresponds to the phosphorylation site of the OmpR protein in *E. coli* is conserved in NrrA (6). However, incubation of NrrA with 100 mM acetyl phosphate did not enhance the DNA-binding activity of NrrA (data not shown). The other OmpR-type response regulator ManR from *Anabaena* sp. strain PCC 7120 and *Synechocystis* sp. strain PCC 6803 is also not activated by incubation with acetyl phosphate (13, 32). It is possible that recombinant NrrA proteins have already been phosphorylated inside the *E. coli* cells or that nonphosphorylated NrrA is able to bind to the upstream re-

gion of *hetR* and phosphorylation of NrrA might be required for the transcriptional activation of *hetR*.

Why does NtcA not directly regulate the expression of *hetR* but does indirectly via NrrA? One idea is that NrrA ensures the more strict regulation of the *hetR* expression than NtcA alone. The 2-OG signal regulates the expression of *nrrA* via NtcA, and some signal other than 2-OG could also regulate the activity of NrrA via a hitherto unidentified histidine kinase (Fig. 6). The possibility that the 2-OG signal is transmitted to NrrA cannot be excluded. Multiple signals could be integrated into NrrA to prevent inappropriate expression of *hetR*.

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