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Role for *hetC* in the Transition to a Nondividing State during Heterocyst Differentiation in *Anabaena* sp.

XUDONG XU† AND C. PETER WOLK*

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824-1312

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Nitrogen-deprived filaments of wild-type or *hetC Anabaena* sp. produce respectively, at semiregular intervals, heterocysts and weakly fluorescent cells. Unlike heterocysts, the latter cells can divide and elongate, producing a pattern of spaced series of small cells. Because a *hetR*::*gfp* fusion is expressed most strongly in the small cells, we propose that these small cells represent a very early stage of heterocyst differentiation. *hetC*::*gfp* is expressed most strongly in proheterocysts and heterocysts.

Cyanobacteria are prokaryotes that are capable of oxygen-producing photosynthesis. Some filamentous species, like *Anabaena* spp., fix dinitrogen under aerobic conditions in specialized cells called heterocysts. These specialized cells differentiate from vegetative cells in response to deprivation of fixed nitrogen. In wild-type *Anabaena* sp. strain PCC 7120 and many other strains, single heterocysts differentiate at semiregular intervals along vegetative filaments, forming a spacing pattern (23). Heterocysts, in which synthesis of biliprotein-containing antennae stops, are much more weakly autofluorescent than vegetative cells (17). A thick envelope, consisting of an outer, polysaccharide layer and an inner, glycolipid layer, is deposited outside the cell wall of developing heterocysts, forming a barrier to the entry of oxygen (23). En route to becoming a heterocyst, the developing cell stops dividing. However, under certain conditions, developing heterocysts can lose their differentiated character and divide (19, 20).

Genes involved in heterocyst differentiation, principally in *Anabaena* sp. strain PCC 7120, have been cloned and characterized. However, mechanisms underlying the progression of differentiation are largely unknown. *hetR*, an autoregulatory gene that encodes a serine-type protease, is required for the initiation of heterocyst differentiation (1, 2, 7, 25). *hetC*, which encodes a member of the family of ATP-binding cassette exporters and is most similar to such exporters of proteins and peptides, is required for an early step in the differentiation of heterocysts. Expression of *hetC*, like that of *hetR*, is under the control of DNA-binding protein NtcA (14). A *hetC* mutant produces a pattern of non- or weakly fluorescent cells (for simplicity, we shall refer to them as weakly fluorescent) but does not form heterocysts distinguishable by bright-field microscopy (12). Expression of *hetR* and of other genes, e.g., *hepA* and *patS*, involved in heterocyst development and physiology has been localized to proheterocysts and/or heterocysts by use of *luxAB*, *gfp*, and *lacZ* as reporters (1, 10, 13, 16, 22). *hepA* is involved in synthesis of the polysaccharide layer (11, 21), and *patS*, which encodes a 17-amino-acid peptide, may regulate the spacing of heterocysts (24). It is not known in which cells *hetC* is expressed. We present evidence that *hetC* is expressed most strongly in proheterocysts and heterocysts and is required for the transition to a nondividing state during heterocyst differentiation.

Methods. *Anabaena* sp. strain PCC 7120 and its derivatives were grown, selected, and induced to differentiate as described elsewhere (12). Plasmids were introduced into *Anabaena* sp. strain PCC 7120 and its derivatives by conjugation (8). Measurement or localization of gene expression using *luxAB* or *gfp* as reporter employed single-crossover homologous recombination. Products of single and double recombinations were selected as previously described (3, 8). All single recombinations and gene interruptions were verified by Southern blotting. When maintained under selection, single recombinants are very stable, and instability is not evident after several days in the absence of selection (4, 9). Luciferase activity of suspensions was measured as relative luminescence units, i.e., arbitrary ATP photometer units (Turner Designs, Sunnyvale, Calif.) normalized to the concentration of chlorophyll in the sample. Observation of the *hetC* pattern was performed with a Zeiss Axiophot photomicroscope (9), as was Nomarski microscopy, or with a Leitz Laborlux S microscope fitted with a G filter system (12). Fluorescence and bright-field images for localization of *gfp* expression were captured with a Hamamatsu

TABLE 1. Luciferase activities of fusions of *luxAB* to genes involved in the development of heterocysts

Strain[a]	Luminescence after the following no. of h of nitrogen deprivation[b]:				
	0	3.5	7	11	22
WT::pRL2356a (*hetC*::*luxAB*)	2.3	59	100	127	104
WT::pRL2215 (*hetR*::*luxAB*)	97	269	337	311	272
DR2134::pRL2215 (*hetC hetR*::*luxAB*)	86	274	284	206	202
WT::pRL573 (*hepA*::*luxAB*)	5.5	7.8	57	161	281
DR2134::pRL573 (*hetC hepA*::*luxAB*)	3.1	2.3	1.6	3.4	8.4

[a] WT, wild type of *Anabaena* sp. strain PCC 7120.
[b] Relative luminescence units per microgram of chlorophyll *a*, calculated from one of three experiments, all of which produced similar results. Standard errors were all less than 30% of the means.

* Corresponding author. Mailing address: MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824-1312. Phone: (517) 353-2049. Fax: (517) 353-9168. E-mail: wolk@msu.edu.
† Present address: Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, China.

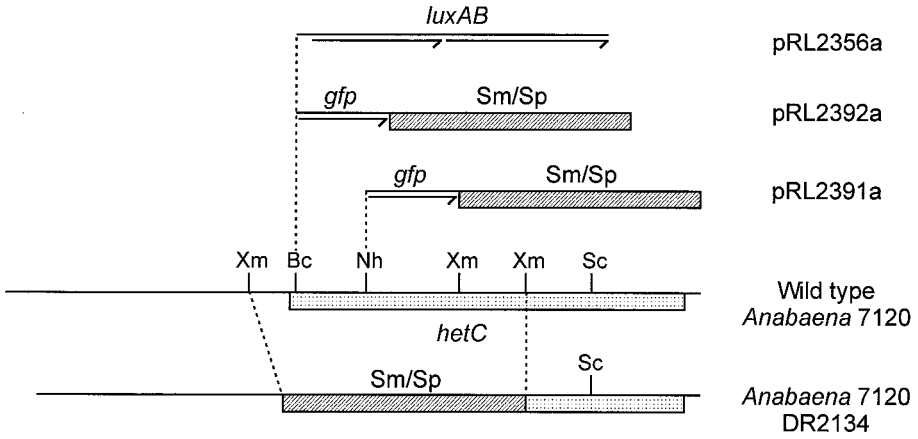

FIG. 1. Constructs for study of expression of *hetC*. In pRL2356a, the *hetC* promoter is cloned as a 2.2-kb *Bam*HI-*Bcl*I fragment from pRL1600 (12) into the *Bgl*II site of pRL579 (9). Promoterless *gfp* was PCR-amplified from pBADgfp (6) with primers CPW135 (5′-TGCCTGCAGGTCG ACTCTAGAGGATC-3′) and CPW195 (5′-TCCCGGGTAGCTAGTTAAGAAGGAGATATACATATGG-3′) and cloned into the *Sma*I site of pUC19 (18); the *omega* cassette (15), cut with *Dra*I, was cloned into the filled-in *Eco*RI site; and the 2.7-kb *Sma*I-*Pvu*II fragment containing the *gfp-omega* cassette was cloned into the filled-in *Nhe*I or *Bcl*I site of *hetC* in pRL1600 and then supplemented with the *Sph*I-cut *sacB-oriT*-Cm^rEm^r fragment from pRL1075 (1), producing pRL2391a and pRL2392a, respectively. *hetC* mutant DR2134 was made by double reciprocal recombination with pRL2134, which was constructed by replacing an *Xmn*I fragment of ca. 2.2 kb in pRL1640 (12) with the *omega* cassette and then transferring a 5.3-kb *Pst*I-*Sac*I fragment between the *Pst*I and *Sac*I sites of pRL271 (1). *hetC* is transcribed from left to right. Restriction sites: Bc, *Bcl*I; Nh, *Nhe*I; Sc, *Sca*I; Xm, *Xmn*I.

Photonics System, model C1966–20 (Photonic Microscopy, Inc., Oak Brook, Ill.), coupled to the Leitz microscope fitted with a Sapphire GFP filter set (Chroma Technology Corp. [Brattleboro, Vt.] Exciter D395/40, dichroic 425DCLP, and emitter D510/40).

***hetC* is expressed most strongly in proheterocysts and heterocysts.** When measured by a transcriptional fusion of *luxAB* to *hetC* at its *Bcl*I site (pRL2356a in Fig. 1 and Table 1), *hetC* is seen to be extensively transcriptionally activated by 3.5 h of nitrogen deprivation, comparable to the time of activation of *hetR* (Table 1) (1) and consistent with qualitative results of primer extension analysis (14). As shown by a *hetC*::*gfp* transcriptional fusion of *gfp* to *hetC* at its *Bcl*I site (in pRL2392a; Fig. 1), *hetC* is expressed most strongly in proheterocysts and heterocysts (Fig. 2). Over the period of image capture, the initially very low fluorescence by vegetative cells increases markedly. Fluorescence of GFP in excess of the background fluorescence of *Anabaena* sp. was not visible when *gfp* was fused to *hetC* at its *Nhe*I site (pRL2391a; Fig. 1 and data not shown), suggesting that transcription of *hetC* may be attenuated between the *Bcl*I and *Nhe*I sites.

The semiregularly spaced, weakly fluorescent cells in *hetC* mutants can divide. Wild-type *Anabaena* sp. strain PCC 7120 forms mature heterocysts by 24 h after nitrogen step-down. When excited with light of 350 to 460 nm, the vegetative cells fluoresce brightly, whereas the heterocysts fluoresce weakly. By 48 h (but not 24 h) of nitrogen deprivation, *hetC* mutants form a pattern of weakly fluorescent cells, often paired, that are indistinguishable from vegetative cells by bright-field microscopy (12). Seeking to determine whether the weakly fluorescent cells would become proheterocysts if nitrogen deprivation was further prolonged, we observed incipient bleaching of many cells after 3 Da at 30 $\mu E\ m^{-2}\ s^{-1}$. To prevent bleaching, the light intensity was decreased after 2 Da from 30 to 3 $\mu E\ m^{-2}\ s^{-1}$ for 3 Da. The weakly fluorescent cells were found to be elongating and dividing, producing daughter cells of de-

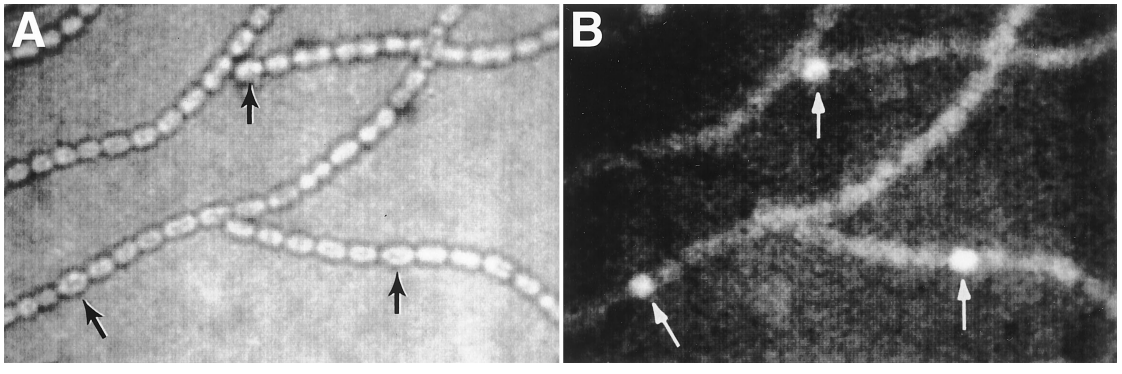

FIG. 2. Bright-field (A) and fluorescence (B) micrographs of filaments of *Anabaena* sp. strain PCC 7120::pRL2392a after 24 h of nitrogen deprivation. *hetC* is expressed most strongly in proheterocysts and heterocysts (arrows).

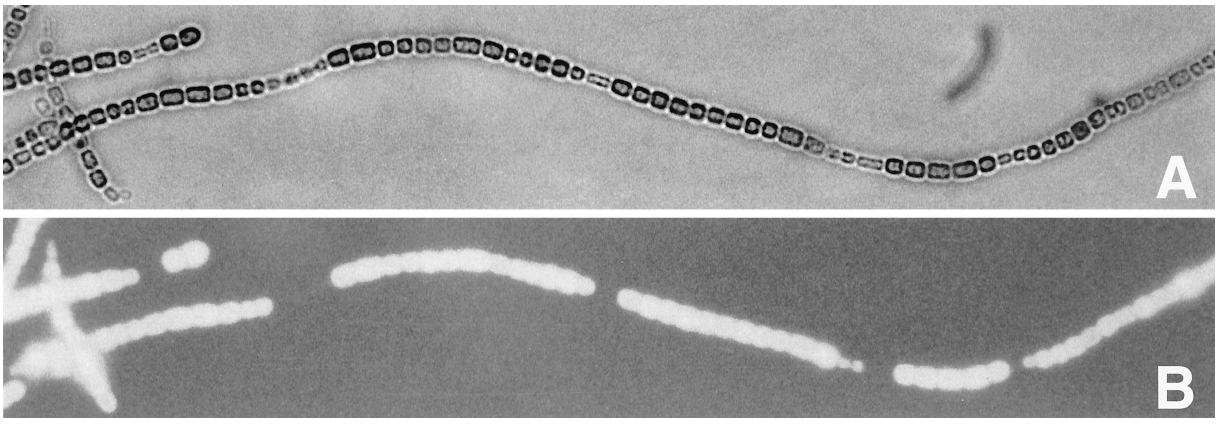

FIG. 3. Nomarski (A) and fluorescence (B) micrographs of a filament of *Anabaena* sp. strain PCC 7120 *hetC* mutant DR2134 (Fig. 1) after incubation without fixed nitrogen at 30 μE m^{-2} s^{-1} for 2 Da and 3 μE m^{-2} s^{-1} for 3 Da, showing a pattern of small, weakly fluorescent cells.

creased size and forming a pattern of spaced series of diminutive, weakly fluorescent cells (Fig. 3).

The weakly fluorescent cells represent a very early stage of heterocyst differentiation. On the basis of the weak fluorescence of the semiregularly spaced cells of a *hetC* mutant, it was suggested that the cells had initiated heterocyst development but stopped at an early stage (12). However, unlike heterocysts or proheterocysts, these cells elongate and divide. RGSGR is a pentapeptide from the C terminus of PatS that acts as an inhibitor of heterocyst formation (24). Addition of the pentapeptide to 1.3 μM at the time of nitrogen step-down prevented the pattern formation characteristic of *hetC* mutants (see also reference 24), while its addition at 48 h prevented the formation of diminutive cells from the weakly fluorescent cells (data not shown). Whereas *hetC* mutant DR1653 (12) showed the phenotype of spaced, weakly fluorescent cells after 48 h of nitrogen deprivation, α41 DR1653, a *hetR hetC* double mutant, like a *hetR* mutant (2, 12), showed no pattern formation (data not shown). These observations are consistent with the idea that the weakly fluorescent cells are related to immature heterocysts.

Plasmids pRL2215, derived from pRL881a (1) by deletion of an *Eco*RV fragment, and pRL573 (22) carry *hetR*::*luxAB* and *hepA*::*luxAB* transcriptional fusions, respectively. By measuring the luciferase activity of recombinants of pRL2215 and pRL573 with *hetC* mutant DR2134 and with wild-type *Anabaena* sp. strain PCC 7120, we found that the *hetC* mutation has little or no effect on the induction of *hetR*, as observed by others (14), but nearly completely blocks that of *hepA* (Table 1). Because *hetR* is specifically induced in developing heterocysts at a very early stage (1, 2, 5, 10), but a *hetC* mutation has little effect on the expression of *hetR*, we used *hetR* to test whether development is initiated in the weakly fluorescent cells. pRL2380a, bearing *hetR*::*gfp*, was constructed by insertion of a 2.7-kb *gfp-omega* cassette (Fig. 1) into the blunted *Sac*I site of pRL881a (1) and was transferred to *hetC* mutant DR2134. Fluorescence microscopy with the Sapphire GFP filter showed strongest expression of *hetR* in the spaced series of diminutive cells (Fig. 4). Using the G filter system, these diminutive cells were found to be only weakly autofluorescent. These observations support the interpretation that the spaced cells in a *hetC* mutant represent a very early stage of heterocyst differentiation. One effect of the *hetC* mutation is that unlike proheterocysts in the wild-type strain, these weakly fluorescent cells do not make the transition to a nondividing state, even after 48 h of nitrogen deprivation. One can imagine that HetC

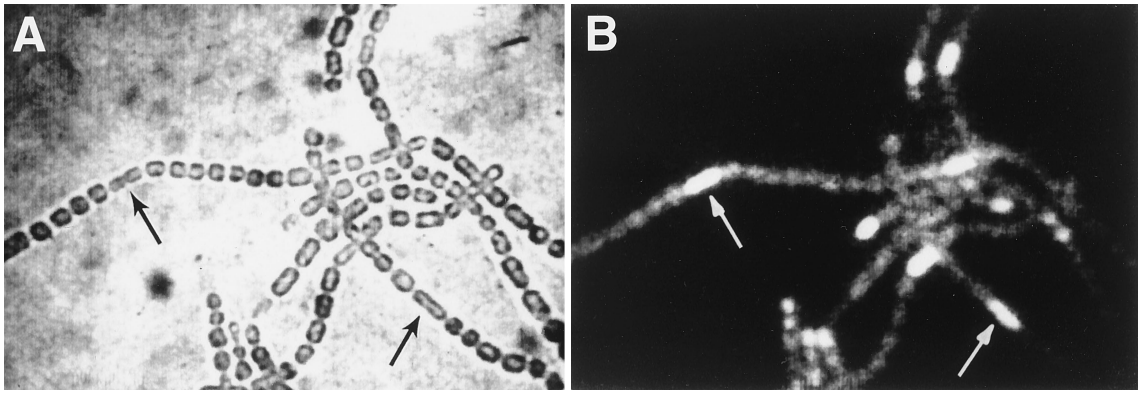

FIG. 4. Bright-field (A) and fluorescence (B) micrographs of filaments of *hetC hetR*::*gfp* strain DR2134::pRL2380a after incubation without fixed nitrogen at 30 μE m^{-2} s^{-1} for 2 Da and 3 μE m^{-2} s^{-1} for 3 Da, showing strongest expression of a *hetR*::*gfp* transcriptional fusion in the dividing and elongating cells (arrows) that are weakly autofluorescent.

is involved in the export of a protein or polypeptide that inhibits the transition to a nondividing state during heterocyst differentiation.

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