Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* 7120

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Anabaena 7120 mutant 216 fails to differentiate heterocysts. We previously identified a 2.4-kb wild-type DNA fragment able to complement this mutant. We show here that the sequence of this fragment contains a single open reading frame (*hetR*), encoding a 299-amino-acid protein. Conjugation of deletion subclones of this fragment into strain 216 showed that the *hetR*-coding region is both necessary and sufficient for complementation of the Het⁻ phenotype. The mutation in 216 is located at nucleotide 535 in the *hetR* gene, converting a serine at position 179 in the wild-type protein to an asparagine in the mutant. Interruption of the *hetR* gene in wild-type cells results in a mutant phenotype identical to that of 216. Both 216 and wild-type cells containing wild-type *hetR* on a plasmid display increased frequency of heterocysts, even on media containing fixed nitrogen. These results suggest that *hetR* encodes a product that is not only essential for but also controls heterocyst development. This putative regulatory protein lacks known structural motifs characteristic of transcription factors and probably acts at a level one or more steps removed from its target genes.

[Key Words: Cyanobacteria; heterocyst; nitrogen fixation; *Anabaena* 7120; regulation; development]

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Cyanobacteria are a diverse family of prokaryotes that carry out oxygenic photosynthesis similar to green plants. Filamentous cyanobacteria grow in long chains of identical vegetative cells when combined nitrogen is available. When fixed nitrogen is limiting, many strains of filamentous cyanobacteria respond by activating genes encoding the subunits of nitrogenase. In heterocystous cyanobacteria such as *Anabaena* 7120, the filaments also undergo patterned cellular differentiation in response to nitrogen limitation. Terminally differentiated cells called heterocysts develop at intervals along the filament, providing the sites at which nitrogen fixation occurs. Heterocysts differ from vegetative cells in a number of functionally important ways. To limit oxygen entry and protect nitrogenase from inactivation, developing heterocysts (proheterocysts) form a new external cell envelope containing inner glycolipid and outer polysaccharide layers. Other structural changes include the reorganization of photosynthetic membranes and the formation of specialized junctions with neighboring vegetative cells. Many biochemical changes occur in the heterocyst, including the inactivation of ribulose biphosphate carboxylase and the oxygen-evolving photosystem II (PSII) photochemical reaction center complex, and the induction of genes involved in nitrogen fixation and its support, such as the oxidative pentose pathway and a heterocyst-specific ferredoxin (Winklenbach and

Wolk 1973; Haselkorn 1978; Stewart 1980; Wolk 1982; Carr 1983; Böhme and Haselkorn 1988).

Changes in intercellular communication are also apparent as a consequence of heterocyst differentiation. In differentiated filaments, carbon compounds from neighboring vegetative cells are transported into heterocysts (Wolk 1968). Glutamine, carrying newly fixed nitrogen in its amide group, is transported from heterocysts to vegetative cells (Meeks et al. 1978). In *Anabaena* 7120, heterocysts are spaced approximately 10–20 cells apart, depending on growth conditions. Heterocysts inhibit the differentiation of adjacent vegetative cells, and as the vegetative interval lengthens new heterocysts form midway between existing ones (Wilcox et al. 1973a). Thus, the heterocyst spacing pattern is maintained during growth on N₂ as nitrogen source. Transport and metabolism of a specific nitrogenous compound such as glutamine has been proposed as the source of this pattern (Haselkorn 1978), but this is unlikely in light of the normal heterocyst spacing patterns that are seen in most mutants defective in nitrogen fixation (W.J. Buikema and R. Haselkorn, unpubl.; Wilcox et al. 1975).

The formation and maintenance of heterocysts is strongly influenced by neighboring vegetative cells and growth conditions. A classic study by Wilcox et al. using filament breakage showed that proheterocysts revert at higher frequencies and at later stages when fewer adja-

cent vegetative cells are present to support them (Wilcox et al. 1973b). This is not surprising considering that vegetative cells act not only as a sink for fixed nitrogen from the heterocysts, but also as the source of fixed carbon to the heterocysts. This carbon serves not only as an energy and reductant source for nitrogen fixation, but as a substrate for the addition of ammonia from the nitrogenase enzyme complex.

Earlier we described the isolation and characterization of chemically induced Fix^- mutants of *Anabaena* 7120, including the complementation of a strain defective in an early step of heterocyst differentiation (Buikema and Haselkorn 1991). Here we describe the characterization of the *hetR* gene of *Anabaena* 7120 that is required for this early step and is capable of inducing multiple adjacent heterocysts when present on a plasmid in wild-type cells.

Results

Sequence of the *hetR* gene

Earlier we had identified and cloned a 2.4-kb partial *Sau3AI* fragment of wild-type DNA able to complement the heterocyst-deficient mutant 216 (Buikema and Haselkorn 1991). A physical map of this fragment is shown in Figure 1. The DNA sequence of the entire 2.4-kb fragment was determined and is shown in Figure 2. The sequence contains one complete open reading frame (ORF) capable of encoding a 299-amino-acid protein of 35 kD, and no transcription termination structures are evident immediately downstream of the ORF. No significant similarities were found using the FASTA program (Pearson and Lipman 1988) to compare the DNA sequence of *hetR* to GenBank (release 64), or its predicted protein

sequence to GenPept (release 64), the translated protein reading frames from GenBank. The expected gene product shows no evidence of extended hydrophobic regions indicative of a signal sequence or membrane localization. The protein contains over 30% charged residues (92 of 299), although the net charge is only -6 , giving an expected pI of 6.37. The codon usage of *hetR* when compared to highly expressed *Anabaena* 7120 genes suggests that it is expressed at a low to moderate level (data not shown). Secondary structure prediction algorithms (Chou and Fasman 1978; Garnier et al. 1978) suggest a predominance of α -helical over β -sheet structures. The *hetR* peptide sequence does not contain significant similarities to common DNA-binding motifs like helix-turn-helix, zinc finger, or helix-loop-helix, as documented in the PROSITE database (Bairoch 1989).

Complementation of mutant 216 using deletion subclones

We sought to determine the minimum genetic requirement for complementation of strain 216 by using some of the deletions constructed for DNA sequencing. Selected deletions were subcloned into the polylinker of the shuttle vector pCCB110 (described in Materials and methods), and the resultant plasmids were transferred via conjugation into strain 216. Figure 1 shows the deletions that were used in this analysis and indicates those that complement 216. The entire ORF is necessary and sufficient for efficient complementation of the mutation in strain 216. Some reduction of complementation efficiency was seen with the two farthest upstream deletions that lacked 270 and 730 bases of pWB216S2.4, but the reason for this is unknown. These deletions may have fused a vector promoter to the *hetR* gene, resulting in a lethal phenotype (see below).

After determining which deletion subclones could still complement strain 216, we observed that the introduction of other subclones with larger deletions resulted in small numbers of Fix^+ colonies on N^- plates after several weeks. It seemed likely that these colonies were the result of recombination between the introduced plasmid and the mutant chromosomal *hetR* gene. We used the frequency of this marker rescue by recombination (as reflected in the number of Fix^+ colonies) together with the end points of those deletion subclones that did not form these colonies to locate the chromosomal lesion in strain 216 (data not shown). These results mapped the mutation to a 160-bp region near the center of the *hetR* gene, as indicated in Figure 1.

A 318-bp region containing the expected mutation in 216 DNA and the corresponding region of wild-type DNA were amplified using the polymerase chain reaction (PCR). The sequences corresponding to the oligonucleotide primers used are shown in Figure 2. Following symmetric amplification, a portion of the double-stranded product was amplified asymmetrically, and the single-stranded product was sequenced. A single nucleotide difference was detected between wild-type and 216 DNA in this region. The guanine at position 1687 in

Figure 1. Physical map of the 2.4-kb insert in plasmid pWB216S2.4 containing the *hetR* region. Only selected restriction sites are indicated. Wide horizontal lines indicate the portions of the nested deletions that were sequenced. The remainder of each line indicates the DNA remaining in that deletion. These deletions were used for complementation tests. If a deletion complemented, the line is marked with a filled circle. The two deletions that complemented poorly are shown marked with stippled circles. Deletions that produced only a few marker-rescued colonies are shown marked with a half-circle. Lines marked with open circles indicate deletions that neither complemented nor gave marker-rescued colonies. The marker-rescue results place the mutation in strain 216 within the 160-bp region shown between the vertical dotted lines. Other deletions used for sequencing but not for complementation tests are not included in the figure.

1 GATCAATAAATTAGTGGGTTGCGGTCAGCTATTATTAGTGAAGTGACCTATTTTGACAGTAATCTTGCTGGTTTGGTACTTCAAGAACTTTAAACATTGCCCAATATTCGGAGT
 121 TTTATTCGTCACCTTTTGGGGCAATTGCTTTTGGTATTGGCAAAATACAAATCCCAACAACCTGCCAATGCAGAAGTTAAACCTTATGACAAAGGACTTATAATCAAAACAAATGTT
 241 TGGTCACTTAGGTAGCAGGAAGAGAATAATTAACTCTGGACTTTCTGGCTCAGTAGTCATAATGGCTTAATGTCAGCAAACCTTGATTTTCCATGGAAAAACCATGGTTATTCAT
 361 CAATCTCGAAATTTTATGCAAAAGAAAAGTTAAGAGTAGATTAATTCATGCAATTTTAAATATATGACAGATAAATAGAAATATTATGTTGTGAGGAAATAAAAGGTATTGCTAAGTT
 481 GGAATTACCTGTCTTATTAGCGTCTATAATTTCCCTCCAGCCAACGACCTACACTGCCATAAGAGCAAAAAAATTGTCAGCATCTCGGCAGATAAAGAACGCTCTATTGATTCAATGGG
 601 CGTTAGTGAGTTTGGCGTAGTCGGCAGGTGACTTTTCAAAAAATCACTTCCTGTTATTCTGGAATAATCGGTCAGCATCTGAGTAAATTAAGTACTCAGGAATAGAGTATTACCTCAAG
 721 GAGAGATTGTGCGATTATAATCTCCCATTTGTTGGCCGTTGTGTCTATCTCTAAATCCGGGTGCAGCCCAAAAGTCTTAAATAGCAAAATTTATTGACGCTATTAAAGAGCAGATAA
 841 GTTCGGATAATAGGAAAGTCCTTGATGGTTACTTATTAGAACCATAAAGTTCGCCAGTAATGACAAATGTTATAAATGAGGAAATTCGGCAGTTATTTGTGATTTTTCGGTAAGA
 961 TACAAGCAGCTAGTAACAACTGTTGCTAGTTATATGCCATAAATACGAGGAATAGCATCTTGACCATTAATCGACTTTTATTTAAACATCAATAGCATTTTGGTGTCAAT
 1081 CTCACCCCTATGGGTGTATACCAACTACTTTTTTTAGACTAATAAGCGTGCTATTCAACTATTTGTAATATGAGTAACGACATCGATCTGATCAAGCGCTCTTGCCCCAGTGAATGG
 M S N D I D L I K R L G P S A M D
 1201 ATCAGATCATGCTATATCTGGCTTTTAGCGCCATGAGGACGAGCGGGCATAGGCATGGAGCATTCTAGATGCAGCAGCAACGCGGCTAAGTGTGCCATTACATGACTTATCTAGAGC
 Q I M L Y L A F S A M R T S G H R H G A F L D A A A T A A K C A I Y M T Y L E Q
 1321 AAGGACAAAACCTCCGGATGACCGGACATTGCACCACTAGAGCCAAAACGGGTCAAAATCATTGTAGAGGAAGTCAGACAAGCCCTGATGGAGGGCAAACTGCTCAAGACCTTAGGTT
 G Q N L R M T G H L H H L E P K R V K I I V E E V R Q A L M E G K L L K T L G S
 1441 CCCAAGAAGCCCGGTTTATTGATTGAGTTTCCCTATGCTCGGATGGAACAGTACCCTTGGATACAGGGCGATCGCGCATTCGCGGTACAAGCCTACAAGTGAAGAAAAAGACAAATTG
 Q E P R Y L I Q F P Y V W M E Q Y P W I P G R S R I P G T S L T S E E K R Q I E
 1561 AGCATAAGTTACCCAGCAATCTTCCGACGCTCAGTTAGTTACTTCTTTGAGTTTTTGGAGTTGATCGAATTTCTCCATAAGCGATCGCAAGAGGATCTGCCCCAGAACATCGCATGG
 H K L P S N L P D A Q L V T S F E F L E L I E F L H K R S Q E D L P P E H R M E
 1681 AGTTAAGCGAAGCCCTGGCAGAGCATATCAAGCGTCGTCGTTTACTCTGGCAGGTGACAAGAATTGATTCTCTTGGGGAATGCCCTTCTATGCCCTGACTCGTCCCTTCTATGCC
 L S E A L A E H I K R R L L Y S G T V T R I D S P W G M P F Y A L T R P F Y A P
 N
 1801 CAGCAGACGACCAAGAGCGGACTTATATTATGGTGAAGATACCGCTCGGTATTTCCGCATGATGAAGACTGGGCAGAAAAGCGGCCAAACGCTATGCGAGCCTTAGAAGAAGTCTGATG
 A D D Q E R T Y I M V E D T A R Y F R M M K D W A E K R P N A M R A L E E L D V
 1921 TGCCACCAGAGCGCTGGGATGAAGCCATGCAGGAAGTGAATATCCGTAACCTGGGCTGATAAATACCAAGTAGGTGGTATCCCCATGATTTTACAAATGGTGTGTTGGTAGAA
 P P E R W D E A M Q E L D E I I R T W A D K Y H Q V G G I P M I L Q M V F G R K
 2041 AAGAAGATTAAAGCAGGAGTGAATAAAGTACTAGTAATGTTTCATTGAGAACTTATGCTAGTGATGACAAATGACTCGGCGAATTAAATTAATCTTGGCATGGCAGATGACACA
 E D *
 2161 GCAGCGATGGGCGGTTTAACTGCTAAAAATTCAGGATTTGATCGCTCTTTGGCGCGATCGTTTATATCAATCAAACCTGGTCAAGTCCCTAGTGAT 2258

Figure 2. Annotated nucleotide sequence of *hetR* and flanking regions and deduced amino acid sequence of the *hetR* gene product. The first available methionine codon in the *hetR* ORF was chosen as the start of translation. The underlined nucleotides indicate the sequences used for priming the PCR employed to identify the mutation in strain 216. The mutated residue at position 1687 is indicated in boldface, as is the wild-type serine that is replaced by asparagine in strain 216.

Figure 2 is changed to an adenine in strain 216, resulting in a single amino acid change in the predicted *hetR* gene product of serine (wild-type) to asparagine (mutant 216).

Insertion cassette mutagenesis of *hetR*

To confirm that inactivation of the *hetR* ORF is sufficient to cause the observed phenotype in strain 216, we constructed a mutation of *hetR* in wild-type *Anabaena* 7120 by insertion of a neomycin-resistance cassette derived from Tn5 as described in Materials and methods. Several independent colonies were isolated and characterized. Every colony showed rapid bleaching and a complete lack of heterocysts when grown under nitrogen-limiting conditions, identical to the phenotype of strain 216. One of these recombinants, WJB313, was confirmed to have the expected genomic arrangement of a single interrupted *hetR* gene by Southern hybridization, using the 700-bp *Hae*II fragment internal to *hetR* as probe (data

not shown). This *hetR* mutant strain grows as well as wild-type and mutant 216 on media containing nitrate or ammonia. Thus, the *hetR* gene does not appear to play a significant role in normal vegetative growth.

Supernumerary heterocysts

In the course of examining strain 216 carrying the complementing wild-type copy of the *hetR* gene in pWB216S2.4, we observed an unusually high frequency of heterocysts in this strain when it was grown under N⁻ conditions. Many of these heterocysts were present in multiples, mostly as pairs but some with as many as five contiguous heterocysts. Typically 10–25% of the total number of cells under these conditions were heterocysts. To confirm that this result is due to the *hetR* gene itself, we examined the phenotypes of 216 carrying each of the deletion derivatives of pWB216S2.4 that had been used to test for complementation of the Het⁻ pheno-

type. In all but one of the cases in which the ORF remained intact, the extra heterocyst phenotype was seen under N^- conditions. The lone exception was a deletion that removed all but 50 bp of DNA upstream to the *hetR* ORF. This plasmid was able to complement 216, but showed a normal number of heterocysts under N^- conditions. Presumably this deletion derivative expresses the plasmid copy of *hetR* at a low enough level to avoid generating excess heterocysts, but at a level high enough to allow complementation.

We also examined the phenotypes of wild-type *Anabaena* 7120 carrying each of the deletion plasmids previously tested in 216. The results paralleled those from 216 with the difference that, since the wild-type strain is Het^+ , normal numbers of heterocysts were seen with

plasmids deleted into the *hetR* ORF from either end. The latter result indicates that the extra heterocyst phenotype is not due to the titration of a factor that binds to DNA outside the *hetR* ORF. Figure 3 shows the typical supernumerary heterocyst phenotype displayed by wild-type *Anabaena* 7120 carrying pWB216S2.4. In this strain grown with nitrate, approximately 5% of the cells look like heterocysts (Fig. 3A). These cells are not as distinct as normal heterocysts and are not functional based on acetylene reduction assays (data not shown). Under N^- conditions, many groups of multiple heterocysts are seen, most of which occur as pairs, but occasionally as groups of three, four, or more cells (Fig. 3B,C). The heterocyst pairs are usually similar in size and appearance, suggesting that they develop synchronously. However,

Figure 3. Light microscopy of *Anabaena* 7120 containing a plasmid carrying the *hetR* gene. *Anabaena* 7120 carrying pWB216S2.4 was grown on BG-11 plates with nitrate (A) or without combined nitrogen (B, C, D). Note the presence of heterocysts in A. Wild-type filaments grown on nitrate typically develop less than 1% heterocysts. In B, C, and D many groups of heterocysts are seen, and the heterocyst frequency is increased with respect to wild-type. B shows the typical appearance of *Anabaena* containing *hetR* on a plasmid. Most of the heterocysts present are single or paired cells. Occasionally higher multiples are seen, as shown in C. Often the heterocysts in these groups have different sizes, indicating that the group formed asynchronously, and one or more of the heterocysts typically appears abnormal. Rarely, heterocysts are seen that appear to be dividing, as shown in D (left center). Bar, 10 μ m.

the higher multiple groups vary in size, often with one or more of the cells appearing abnormal, suggesting that the group develops asynchronously. Occasionally pairs of heterocysts are seen in which the two cells are incompletely separated or appear to be dividing (Fig. 3D). The appearance of these heterocysts suggests that dividing cells may occasionally be induced to form heterocysts before cell division is complete.

The excess heterocyst phenotype of *Anabaena* strains containing a plasmid with an intact copy of the *hetR* gene, such as pWB216S2.4, was typically unstable. Older cultures showed a high frequency of plasmid loss. When maintained on N⁺ plates, strain 216 carrying pWB216S2.4 often formed colonies that were Fix⁻ Het⁻ on return to N⁻ medium. A continuous liquid culture of wild-type *Anabaena* 7120 containing pWB216S2.4 grown for 3 weeks under neomycin selection with ammonia as nitrogen source yielded approximately 50% Fix⁻ Het⁻ mutant colonies. *Anabaena* 7120 containing pWB216S2.4 grows more slowly on selective plates with fixed nitrogen than strains containing an inactive *hetR* plasmid, and grows very slowly on selective N⁻ plates. Although the nature of these mutations is being studied further, it is likely that the presence of extra copies of *hetR* results in a strong depression of growth rate. Inappropriately formed heterocysts directly reduce the growth rate of filaments by being terminally differentiated, and indirectly by acting as a sink for fixed carbon from neighboring cells. Thus, it is probable that mutations, gene conversions, or recombination events that inactivate the extra copies of *hetR* or other genes required for differentiation are selected under these conditions.

Expression of *hetR*

The timing and level of expression of the *hetR* gene was determined by Northern blot analysis. Total RNA was prepared from wild-type *Anabaena* 7120 at 6-hr intervals after induction by removal of fixed nitrogen. A blot of this RNA was probed with the internal 0.7-kb *Hae*II fragment of the *hetR* gene (see Fig. 1) with the results shown in Figure 4. At zero time, only weak hybridization is seen in two bands of 1.4 kb and 1.9 kb, indicating that *hetR* is transcribed at a low level during growth under nitrogen-replete conditions. From 6 to 18 hr post-induction, the level of *hetR* RNA increases from three- to fivefold, decreasing slightly after 18 hr. Both the 1.4-kb and 1.9-kb bands increase uniformly in intensity, and a very faint band of 2.4 kb appears. This result indicates that induction of *hetR* occurs very early during heterocyst development.

The presence of multiple bands on the Northern blot suggests that *hetR* is transcribed from multiple promoters or that the mRNA is processed. The 1.4-kb and 1.9-kb major transcripts contain 0.5 kb and 1.0 kb more than is necessary to encode the 900-bp *hetR* ORF. Because no significant ORFs are apparent within 1 kb upstream of *hetR*, it is possible that both transcripts originate from this region without encoding additional genes. Although

Figure 4. Hybridization of *hetR* to total RNA isolated from *Anabaena* 7120 induced by nitrogen starvation. The 700-bp *Hae*II fragment internal to *hetR* (Fig. 1) was used to probe a Northern blot of RNA isolated from *Anabaena* 7120 induced for heterocyst differentiation at the times shown (in hours). Sizes are based on glyoxalated RNA standards.

this possibility requires very long 5'-untranslated regions, such lengths have been observed in other *Anabaena* transcripts, such as the *rbcLS* operon (Nierzwicki-Bauer et al. 1984) and the *Anabaena* 7120 *sigA* gene, for which B. Brahamsha has mapped the 5' end of one transcript approximately 800 bp upstream of the gene (B. Brahamsha, unpubl.). Alternatively, the transcripts may extend downstream from *hetR*. No additional ORFs are present in the 200 bp 3' to *hetR* that have been sequenced.

Distribution of *hetR* in cyanobacteria

As a means of correlating the presence of *hetR* with heterocyst formation, it was of interest to determine the distribution of the *hetR* gene in various groups of cyanobacteria. An internal 0.7-kb *Hae*II fragment (Fig. 1) of the *hetR* gene was used to probe a Southern blot of chromosomal DNAs from 24 strains of cyanobacteria, under conditions of high stringency. We included heterocystous, filamentous nonheterocystous nitrogen-fixing, and single-celled nonfixing strains in the comparison. As shown in Figure 5, DNA hybridizing to the *hetR* gene is present in all filamentous cyanobacteria examined but is not present in the two single-celled cyanobacterial strains examined. To test for nitrogen fixation ability in the tested strains, the blot was reprobed with the *nifH* gene region of *Anabaena* 7120 found in pAn154.3 (Rice et al. 1982). The presence of a *hetR* analog in all of the filamentous strains coincides with the presence of DNA hybridizing to the *nifH* gene. Conversely, no hybridization to *nifH* was seen for the single-celled strains (data not shown).

In general, the intensity of hybridization to the *hetR*

Figure 5. Hybridization of *hetR* to genomic DNA from several classes of cyanobacteria. Approximately 2 μ g of genomic DNA restricted with *Hind*III was used from each of the indicated cyanobacterial strains, and the Southern blot was probed with the 700-bp *Hae*II fragment internal to *hetR* (Fig. 1). The smears seen in several lanes are caused by partially sheared DNA samples. With the exception of *Anabaena* 7118, all of the *Anabaena*, *Nostoc*, and *Calothrix* strains are filamentous heterocystous cyanobacteria. The *Phormidium*, *Plectonema*, and *Oscillatoria* strains are filamentous, nonheterocystous, anaerobically nitrogen-fixing cyanobacteria. The two *Synechocystis* strains are unicellular non-nitrogen-fixing cyanobacteria.

probe is correlated with the relatedness of the strains examined to *Anabaena* 7120. Strains that are nearly identical to 7120, such as 7118, 7119, and M-131, show the strongest hybridization and similarly sized bands. More distantly related cyanobacteria, such as the non-heterocystous *Phormidium*, *Plectonema*, and *Oscillatoria* strains, show a lower level of hybridization and variable band sizes. In these cyanobacteria, a *hetR* analog cannot be involved in heterocyst formation, but it may function in the patterned development of nitrogen-fixing cells. Further work is required to determine if *hetR* analogs always accompany nitrogen fixation capability, both by expanding the number of strains tested and by

testing other categories of cyanobacteria, such as filamentous non-nitrogen-fixing species and single-celled nitrogen-fixing species.

Discussion

The *hetR* gene appears to be a novel regulatory gene. It contains no sequence similarity to *rpoD* or any other sigma factor-like genes from *Anabaena* (B. Brahamsha, pers. comm.). The *hetR* protein sequence contains no apparent DNA-binding domains, such as zinc finger or helix-turn-helix, indicative of transcriptional activators. There is no sequence similarity to the protein ki-

nases or their DNA-binding substrates that activate many environment-sensitive genes in bacteria (Kustu et al. 1989). There have been no difficulties in subcloning the *hetR* gene in *Escherichia coli*, suggesting that there is no interaction between its product and the transcription apparatus of *E. coli*. The identification of the probable mutation of the *hetR* product in strain 216 resulting in a change from serine to asparagine may be useful in the eventual determination of its function.

The *hetR* gene appears to be transcribed at a low level under N^+ conditions, but it is not essential for vegetative growth because a null mutation in *hetR* is not lethal. Upon transfer to N^- conditions, the abundance of the *hetR* transcript increases rapidly within 6 hr, consistent with an early role for the *hetR* protein.

hetR gene function in *Anabaena* 7120 is similar in some respects to that of the *whiG* gene of *Streptomyces coelicolor* (Chater et al. 1989) and the *brlA* gene of *Aspergillus nidulans* (Adams et al. 1988). Interruption of the *whiG* gene prevents sporulation but has no effect on vegetative growth. Likewise, *brlA* mutants are defective in the later stages of conidiophore development, but not in vegetative growth. The sequence of the *whiG* product is similar to that of several *Bacillus subtilis* sigma factors but not to that of four other *S. coelicolor* sigma factors. Greatest similarity is not with *B. subtilis* sigma factors required for sporulation, but with σ^D (σ^{28}), used for transcription of genes involved in motility and chemotaxis (Helmann et al. 1988). In contrast, *BrlA* contains two zinc finger motifs, indicative of transcriptional regulatory function (Adams et al. 1988). Extra copies of the wild-type *whiG* gene result in a hypersporulation phenotype that can be reversed by introduction of multiple copies of a promoter recognized by σ^D . Similarly, mischeduled expression of *brlA* results in cessation of vegetative growth and the function of conidiospores.

The presence of extra copies of *hetR* in *Anabaena* 7120 has deleterious consequences. Under N^- conditions, it leads to supernumerary heterocysts, which may develop as arrays of two or more adjacent heterocysts. Under other circumstances it also appears to be detrimental, resulting in the accumulation of mutations in the cloned *hetR* gene or possibly in other genes that participate in the commitment of heterocysts to differentiation. In the isolation of wild-type genes by mutant complementation, the isolated *hetR* gene was occasionally mutated by the time it was recovered in *E. coli*. Presumably these mutations arose by genetic exchange with the mutated chromosomal copy of *hetR*. Because the same excessive heterocyst phenotype is observed in wild-type *Anabaena* 7120 after introduction of *hetR* on a plasmid, it may be possible to identify new genes whose products are required for differentiation by selecting Fix^- survivors from such plasmid-containing strains.

The cellular interactions governing the pattern of heterocyst differentiation have been reviewed recently (Wolk 1989). Based on the early observations of Wolk that mechanical disruption of filaments led to an increase of heterocyst frequency (Wolk 1967), subsequent work of Wilcox on mechanical destruction of individual

proheterocysts (Wilcox et al. 1973b), and that of Meeks on the metabolic path of nitrogen following fixation of N_2 (Meeks et al. 1978), the following picture of pattern formation emerged: heterocysts are the source of an inhibitor of differentiation, possibly glutamine or a derivative, that diffuses along the filament from cell to cell and is metabolized by vegetative cells. When the level of inhibitor falls below a threshold in a candidate cell, that cell initiates differentiation. A certain minimum number of adjacent vegetative cells must remain to provide support for the developing heterocyst, otherwise it will cease development and revert to a vegetative cell. In strains such as *Anabaena* 7120 that do not typically form multiple heterocysts under free-living conditions, heterocysts that begin to form as pairs compete with each other until one becomes a heterocyst and the other regresses to a vegetative cell.

Our results shed no light on the questions of candidate cells or the nature of the inhibitor. With regard to the mechanism by which *hetR* regulates differentiation, the *hetR* gene product could act positively in developing cells or negatively in vegetative cells. Since mutant 216 and the null mutant WJB313 do not differentiate under any conditions, we believe that the *hetR* product acts positively in developing cells. These mutants bleach rapidly under nitrogen starvation conditions, so *hetR* is not required for the activation of the protease(s) that digest the phycobiliproteins (Wood and Haselkorn 1980). The *hetR* gene could encode a different protease (with serine-179 as part of its active site) but the sequence data base searches have thus far failed to find similarity with known proteases.

The supernumerary heterocyst phenotype of the multicopy *hetR* strains is consistent with positive action of the gene product. One could envision the wild-type *hetR* gene product as a necessary activator of differentiation unless it binds a nitrogen-containing inhibitor, such as glutamine. Inactivation or titration of the *hetR* gene product with inhibitor would result in the absence of activation ability. In the extra-copy *hetR* strain there would be insufficient inhibitor to titrate all the *hetR* product in many of the cells, and these would differentiate. Effectively, the extra copies of *hetR* would raise the threshold of inhibitor concentration below which differentiation is initiated. Thus, the differentiation unit would sometimes become not a single cell, but a group of cells, and the spacing between heterocysts would narrow. In support of this idea is the evidence that heterocyst numbers in strains containing extra copies of *hetR* are correlated with the level of inhibition of heterocyst development afforded by different nitrogen sources. The lowest frequency of heterocysts (~5%) is seen when ammonia is used as the nitrogen source, which is correlated with the ability of ammonia to repress heterocyst development completely in wild-type *Anabaena* 7120. During growth with nitrate, extra-copy *hetR* strains produce 5–10% heterocysts, consistent with the observation that wild-type *Anabaena* is not completely repressed by nitrate, typically forming up to 1% heterocysts in our cultures. Finally, under completely derepressing conditions

when no fixed nitrogen is present, extra-copy *hetR* strains produce supernumerary heterocysts at 10–25%, compared to the typical 5–10% frequency of heterocysts in wild-type *Anabaena*.

There are alternative hypotheses for the role of the *hetR* gene product in heterocyst differentiation. Even though *hetR* does not show significant sequence similarity to known sigma factors, its effect on heterocyst differentiation could be explained if the *Anabaena* cells are both dependent on its function for differentiation and sensitive to the levels of its product, as might occur if *hetR* interacted with RNA polymerase as a transcriptional activator for differentiation. The inappropriate expression of such an activator might lead to the differentiation of cells at an improper place in the cell cycle, affording an explanation for the occasional appearance of tightly apposed and incompletely divided heterocysts.

Another possibility is that the *hetR* gene product might be more indirectly involved in differentiation, perhaps by measuring the ratio of fixed carbon to nitrogen in the cell. Here one could envision the lack of *hetR* to result in the “sensing” of a low carbon to nitrogen ratio. Because it has been shown that heterocysts require the support of a minimum number of adjacent vegetative cells (Wilcox et al. 1973b), it is likely that filaments with low fixed-carbon reserves will not effectively differentiate heterocysts. Thus, *hetR* mutant cells might act as if chronically starved for fixed carbon and not differentiate heterocysts under any conditions. Conversely, in the presence of excess gene product, an abnormally high ratio of carbon to nitrogen might be sensed, resulting in the production of excessive heterocysts. Such a general function for *hetR* might explain the presence of *hetR*-hybridizing DNA in nonheterocystous nitrogen-fixing strains of cyanobacteria.

Multiple heterocysts have been seen before in cyanobacteria. Supernumerary heterocysts appear when *Anabaena* is grown in the presence of subinhibitory concentrations of rifamycin (Wolk and Quine 1975) or in the presence of the tryptophan analog 7-azatryptophan (Mitchison and Wilcox 1973). In neither of these cases has a mechanism been proposed for the observed behavior. Mutants of *Anabaena cylindrica* have been described that produce supernumerary grouped heterocysts constitutively (Wilcox et al. 1975). In several symbioses, the frequency of heterocysts is considerably elevated over the frequency seen in the free-living state (Hill 1975; Peters et al. 1980). In these cases and in our experience with extra-copy *hetR*, the pattern of heterocyst formation is altered, and multiple adjacent heterocysts are often formed along the filament. Heterocysts are often more closely spaced in general, leading to an overall increase in heterocyst numbers. It is plausible that relatively simple changes in signal generation or reception can account for these observations.

Materials and methods

Strains and culture conditions

Many of the strains of cyanobacteria mentioned here have been

previously described (Rippka et al. 1979; Schneider and Haselkorn 1988; Buikema and Haselkorn 1991). Other strains were received from the following sources: *Anabaena* 7118 Rev, a Fix⁺ revertant of PCC 7118, and *Synechocystis* PCC 6803 from R. Rippka at the Institut Pasteur; *Nostoc* 7911 Mac R1 and R2, two independent Fix⁺ revertants of *Nostoc* 7911 Mac, and *Nostoc* 7801, from J. Meeks at the University of California at Davis; *Anabaena* M-131 and *Nostoc* ATCC 29107 from C.P. Wolk at Michigan State University; *Anabaena* 77S15 from C. Franche at the Institut Pasteur; *Anabaena* CA ATCC 33047 from F.R. Tabita at Ohio State University; and *Synechocystis* PCC 6714 from F. Jøset at the Faculté des Sciences Luminy, Marseille.

For liquid culture, *Anabaena* strains were grown in a modified Kratz and Myers medium C (K&M) or BG-11 medium (Kratz and Myers 1955; Rippka et al. 1979). The modified K&M medium contained 1.125 mM Na₂HPO₄ and K₂HPO₄ each in place of the Na₂HPO₄, and typically contained 3–5 mM MOPS buffer (pH 7.5). For N⁺ growth, K&M was supplemented with 2.5 mM (NH₄)₂SO₄ and BG-11 with 17.6 mM NaNO₃. For growth on plates, 1.3% agar (BBL purified) was added prior to autoclaving. Cultures were typically grown under 30–40 μE/m²/sec cool white fluorescent lighting at 25–30°C in the presence of 2% CO₂ (large-scale liquid cultures were bubbled with a 2% CO₂-air mixture). For selective growth of *Anabaena* 7120 strains, media were supplemented with 25–30 μg/ml neomycin. Mid-log phase cells refer to cultures containing 2–6 μg/ml of chlorophyll corresponding to 0.7 × 10⁷ to 2.0 × 10⁷ cells/ml.

E. coli strains were typically grown in Luria broth (LB) (Maniatis et al. 1982) for liquid culture, and LB solidified with 1.3% agar for plate culture. For selective growth, media were supplemented with antibiotics at the following concentrations: 100 μg/ml ampicillin or carbenicillin, 50 μg/ml kanamycin, and 10 μg/ml chloramphenicol. For plasmid isolation, *E. coli* strains were grown in LB supplemented with M9 salts (Maniatis et al. 1982), 2 g/liter glucose, 1 g/liter sodium succinate, and the appropriate antibiotic.

Molecular biological techniques

General molecular biological techniques were as described (Maniatis et al. 1982). Plasmid isolation from *E. coli* was by the alkaline lysis method.

Sequencing templates were prepared from the insert of pWB216S2.4 by digestion with *Pst*I, resulting in a 3.2-kb fragment containing the 2.4-kb *Sau*3AI insert joined to the 3' end of the *neo* gene from pDUCA7 (Buikema and Haselkorn 1991). The 3.2-kb fragment was ligated into the *Pst*I site of pUC19, and two plasmids containing the insert in opposite orientations were obtained. One of these, pWB19-7, was used for sequencing without further modifications. The other plasmid was digested with *Sma*I to release most of the fragment of pDUCA7, followed by religation to form pWB19-12. Several sequencing templates (see below) with deletions of varying lengths 5' or 3' to the *hetR* ORF were selected for subcloning into an *Anabaena* shuttle vector. The inserts from these deletion plasmids were removed using *Pvu*II and ligated into *Sma*I-digested pCCB110. pCCB110 is a shuttle vector based on pRL25C (Elhai and Wolk 1988) which has had the polylinker from pIC20R (Marsh et al. 1984) ligated into the *Eco*RI site (C. Bauer, pers. comm.).

Northern and Southern hybridizations

DNA of many of the cyanobacterial strains was provided by Martin E. Mulligan (Memorial University of Newfoundland). DNA was prepared from the remaining strains as previously

described (Golden et al. 1985). A Northern blot of total RNA (10 µg per lane) from *Anabaena* 7120 isolated at 6-hr intervals from an N⁻ induced culture as described (Golden et al. 1987) was obtained from Bianca Brahamsha. Northern blot analysis was performed as described (Maniatis et al. 1982). Southern blot analysis was performed as described (Reed and Mann 1985) using 1–2 µg of DNA per lane.

DNA sequence analysis

Sequencing deletions were constructed using an Erase-a-Base kit from Promega Biotech on *Sst*I- and *Bam*HI-digested pWB19-7 and *Sst*I- and *Sma*I-digested pWB19-12. Sequences were determined by the chain-termination method (Sanger et al. 1977) using Sequenase (United States Biochemical Corporation) on double-stranded template DNA using a modification of a published method (Kraft et al. 1988). Essentially the ethanol precipitation step after RNase digestion was modified to use 0.6 volumes of isopropanol at room temperature to avoid precipitating RNA oligonucleotides, and the subsequent PEG precipitation was omitted. Buffer gradient sequencing gels were prepared and run as described (Sheen and Seed 1988). Both strands were completely determined, and the sequence is available from GenBank under accession number M37779. DNA sequence was analyzed using the University of Wisconsin Genetic Computer Group's Sequence Software Package and programs written by one of us (W.J.B.) for the Apple Macintosh.

PCR techniques

Techniques for the amplification of selected DNA sequences by polymerase chain reaction (PCR) were essentially as described in the manual accompanying the GeneAmp kit (Perkin Elmer Cetus). A 318-bp region from strain 216 and wild-type *Anabaena* 7120 DNA was amplified using the following two 26-base flanking oligonucleotides: 5'-CCAGGGCGAT CGCGC-ATTCC CGGTAC-3' and 5'-GTCCGCTCTT GGTCTCTGC TGGGGC-3'. Following symmetric amplification, approximately 0.1 µg of the double-stranded product was asymmetrically amplified using a 50 : 1 ratio of one oligonucleotide to the other. Both strands were amplified by reversing the ratio of oligonucleotides. The resultant single-stranded DNA was separated from excess oligonucleotides by passage through a Sephacryl 300 column and sequenced as described above using the appropriate 26-base oligonucleotide primer.

Construction of the *hetR* insertion mutant WJB313

Anabaena strain WJB313, in which the genomic *hetR* gene is interrupted by a neomycin-resistance cassette, was constructed as follows. The *neo* gene of Tn5 driven by the *Anabaena* 7120 *woxA* promoter (Borthakur and Haselkorn 1989) from pDB13 (Borthakur et al. 1990) was removed using *Sma*I, *Hind*III, and *Sca*I. The *woxA* gene encodes the water-splitting protein of photosystem II; its promoter is constitutive in *Anabaena* 7120. Following end repair with Klenow fragment, this DNA was ligated into the end-repaired *Xba*I site of the *hetR* gene in pWB19-12 to form pWB19-12K. The entire interrupted *hetR* gene was isolated by digestion of pWB19-12K with *Sst*I and *Sal*I and ligated into *Sst*I- and *Xho*I-digested pRL271 [which contains the *B. subtilis* *sacB* gene (Cai and Wolk 1990), and was kindly provided by Y. Cai and C.P. Wolk] to form pWB19-12KS. After transformation of *E. coli* MC1061, resultant colonies were verified for sensitivity to 5% sucrose on LB plates due to the presence of the *sacB* gene. pWB19-12KS was then used to transform MC1061/pRL528 for conjugation into wild-type *Anabaena*

7120, as described above. Single recombinant colonies in which the entire pWB19-12KS had integrated into the genomic copy of *hetR* were identified after selection on BG-11 N⁺ plates containing 30 µg/ml neomycin for 2–3 weeks. Double recombinants in which a single interrupted copy of *hetR* remained in the genome were isolated by placing $\sim 5 \times 10^7$ single recombinant cells on BG-11 N⁺ plates containing 5% sucrose and 30 µg/ml neomycin. Several single colonies were selected and seen to have the same phenotype as strain 216. Southern hybridization to genomic DNA digests from the presumptive single and double recombinants demonstrated the expected restriction patterns, indicative of two copies of the *hetR* gene (one interrupted) and plasmid sequences in the single recombinants, and one copy of the interrupted *hetR* gene in the double recombinants (data not shown).

Microscopy

Photomicroscopy of *Anabaena* cells was performed on a Zeiss Axiophot microscope equipped with phase-contrast and Nomarsky (DIC) optics. Photomicrographs were taken using Kodak Technical Pan film at 50 ASA.

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Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* 7120.

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