Developmental regulation and spatial pattern of expression of the structural genes for nitrogenase in the cyanobacterium *Anabaena*

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Depriving the cyanobacterium *Anabaena* of fixed nitrogen induces the differentiation of heterocysts at intervals along its filaments. To test whether the oxygen-deficient conditions believed to prevail within mature heterocysts are sufficient, in the absence of fixed nitrogen, to elicit the expression of nitrogenase, *P_{nifHDK}* was fused transcriptionally to *luxAB* (encoding luciferase). Expression, monitored from individual cells as light emission, was localized (with a resolution of $\sim 1\ \mu\text{m}$) to differentiated cells, whether or not oxygen was present. *Anabaena* PCC 7118 is a heterocystless mutant strain that is known to fix nitrogen when deprived of combined nitrogen under anaerobic conditions. Three lines of evidence indicate that the mutant has retained the ability to develop a pattern despite its inability to make heterocysts. First, morphologically distinct cells appear at non-random intervals when filaments are starved of nitrogen. Second, these cells, like heterocysts, have little or no phycocyanin-dependent fluorescence. Third, nitrogen-starved filaments fragment, with fragment lengths similar to the spacing normally seen between heterocysts. Expression of *P_{nifHDK}-luxAB* was largely confined to differentiated cells in the mutant as in the wild-type strain. These results provide evidence for a causal relationship between development and transcriptional events in *Anabaena*.

Key words: differentiation/heterocyst/luciferase/*nif* gene expression/transcriptional fusion

Introduction

Anabaena is a filamentous photosynthetic prokaryote capable of patterned differentiation in response to starvation for nitrogen (Wolk, 1982). Terminally differentiated cells, called heterocysts, appear at semi-regularly spaced intervals after filaments are shifted to a nitrogen-free medium (Wolk and Quine, 1975; Bradley and Carr, 1976). Cells that differentiate into heterocysts gain the ability to reduce nitrogen gas but lose the ability to fix carbon dioxide, and the growth of filaments therefore depends upon an exchange of metabolites between differentiated heterocysts and photosynthetically competent vegetative cells. This separation of function is necessary since nitrogenase is extremely sensitive to oxygen, which is produced by photosynthesis.

Intercellular communication is also important in establishing the pattern of heterocysts within a filament. Many, if not all, of the cells in a filament have the potential

to differentiate into heterocysts. The placement of heterocysts can be altered by surgical manipulation of the filaments (Wolk, 1967; Wilcox *et al.*, 1973; Wolk and Quine, 1975; van de Water and Simon, 1982) or by modulation of the light intensity (Adams and Carr, 1981). Evidently, the developmental fate of a cell is controlled at least in part by its position, not solely by its lineage, and in this respect pattern formation in *Anabaena* appears similar to that seen in certain cases of eukaryotic development (Doe and Goodman, 1985). The relative simplicity of development in this prokaryote has prompted several laboratories to devise tools intended for elucidating the molecular mechanisms underlying pattern formation in *Anabaena*. Many mutants altered in heterocyst differentiation or spacing have been isolated (Wilcox *et al.*, 1975; Currier *et al.*, 1977; Wolk *et al.*, 1988), and specific genes can be tagged or modified by homologous recombination (Golden and Weist, 1988) with DNA introduced by conjugation (Wolk *et al.*, 1984).

The molecular mechanisms emerging from the study of the sporulation of *Bacillus subtilis* (Losick *et al.*, 1988) can be taken as a paradigm to help one to understand many instances of bacterial differentiation (Kroos and Kaiser, 1987; Newton, 1988; Chater, 1989). The essential feature of this paradigm is that the ordered sequence of events that typifies differentiation is regulated at the level of transcription by sigma factors (subunits of RNA polymerase) and other regulatory proteins whose appearance is often also transcriptionally regulated. It is tempting to apply the *Bacillus* paradigm to *Anabaena*, because proteins appear and disappear at characteristic times during heterocyst differentiation (Fleming and Haselkorn, 1974). However, there is little evidence that the timing and localization of this process is regulated transcriptionally. The strongest argument for the transcriptional regulation of differentiation in *Anabaena* is that the *nifHDK* operon, encoding nitrogenase, is induced at the time that heterocysts reach maturity (Rice *et al.*, 1982) and a DNA rearrangement of the operon that is required for proper expression takes place only in heterocysts (Golden *et al.*, 1985; Golden and Wiest, 1988). If, in accord with the *Bacillus* model, the expression of this operon relies directly on a gene product that is specific to heterocyst differentiation, then we say that expression of the operon is under developmental control.

Alternatively, the *nifHDK* operon may be under environmental control, i.e., induced by the absence of fixed nitrogen under anaerobic conditions (believed to prevail within mature heterocysts; Murry and Wolk, 1989) and otherwise independent of the state of differentiation, as seen in the regulation of *nif* genes in *Klebsiella pneumoniae* (Gussin *et al.*, 1986). In this well studied case, *nif* genes are regulated by *nifA*, a transcriptional activator. The level and activity of NifA protein are increased by starvation for combined nitrogen and by anaerobic conditions. In this way, *nif* genes are turned on only when fixed nitrogen is limiting

and when nitrogenase will not be inactivated by oxygen. Similar regulatory mechanisms appear to operate in other nitrogen-fixing organisms (Masepohl *et al.*, 1988; Merrick, 1988; Kullik *et al.*, 1989). If the *nif* genes of *Anabaena* are also regulated in this fashion, then their transcriptional activation at the end of heterocyst differentiation cannot be taken as evidence of a transcriptionally regulated program of development. Indeed, it is not known whether the expression of *nifHDK* has any connection to the development of pattern.

One means of resolving whether the *nifHDK* operon of *Anabaena* is regulated by environmental conditions, as in *Klebsiella*, or as part of a developmental program is to determine whether the operon is active in all cells or only in certain cells of anaerobically induced filaments. Under these conditions, immature heterocysts appear, lacking the glycolipid layer required to protect nitrogenase from atmospheric oxygen (Rippka and Stanier, 1978; Murry and Wolk, 1989). Several groups have attempted to ascertain the location of nitrogenase in anaerobically induced filaments (see Discussion), but no consensus has been reached. Perhaps the most compelling argument favoring environmental control of *nifHDK* expression is the existence of a mutant strain of *Anabaena* (PCC 7118) that does not differentiate in response to nitrogen deprivation, as judged by bright-field microscopy, but does fix nitrogen under anaerobic conditions (Rippka and Stanier, 1978).

We have analyzed expression of the *nifHDK* operon in a wild-type *Anabaena*, PCC 7120, and in mutant strain PCC 7118. Fusion of the promoter of this operon to *lacZ* (encoding β -galactosidase) and to *luxAB* (encoding bacterial luciferase) enabled us to monitor gene expression in time and space. Gene fusions to luciferase have been used to localize transcriptional activity in plants (Ow *et al.*, 1986) and bacterial colonies (Schauer *et al.*, 1988). We have extended this technique to a resolution of $\sim 1 \mu\text{m}$, enabling us to observe the pattern of transcription within a bacterial filament.

Results

Localization of *P_{nifHDK}* activity in *Anabaena* PCC 7120 during aerobic and anaerobic inductions

Since nitrogenase components, encoded by *nifHDK*, are present under aerobic conditions only in heterocysts (Peterson and Wolk, 1978; Bergman *et al.*, 1986), one would anticipate that transcription of these genes is similarly localized. *P_{nifHDK}* was fused to *luxAB*, encoding luciferase, and the resulting plasmid (pRL562; see Figure 1) was transferred into *Anabaena* PCC 7120. Hybridization analysis (Southern, 1975) confirmed that this plasmid and other plasmids lacking pDU1 used in this study integrated into the chromosome at a site directed by the cloned region of homology (data not shown). Figure 2A and B shows light emission from a filament of the resulting strain, which was grown continuously under air in the absence of combined nitrogen. As expected, little light is seen except from heterocysts, which can be identified by their thickened walls, round shapes, polar plugs and pinched contacts with adjacent cells. The opposite pattern of luciferase activity (Figure 2C and D) is observed in *Anabaena* carrying *luxAB* fused to *P_{rbcLS}*. This promoter drives expression of genes that encode ribulose biphosphate carboxylase, a photosynthetic

enzyme found in vegetative cells but not in heterocysts (Winkenbach and Wolk, 1973; Codd and Stewart, 1977).

The same strains bearing promoter–luciferase fusions

Fig. 1. Vectors and promoters used to construct promoter–reporter transcriptional fusions. All vectors contain polylinkers (+++) upstream from promoterless genes encoding luciferase (*luxAB*) or β -galactosidase (*lacZ*). The *npt* gene encodes neomycin phosphotransferase, which confers resistance to neomycin and kanamycin. The region labeled pDU1 comes from the plasmid of that name from *Nostoc* PCC 7524 (another nitrogen-fixing cyanobacterium) and enables the vector to propagate in *Anabaena* PCC 7120 (Wolk *et al.*, 1984). The promoters shown are taken from DNA isolated from *Anabaena* PCC 7120. *nifH* and *nifD* encode subunits of nitrogenase, *rbcL* encodes the large subunit of ribulose biphosphate carboxylase/oxygenase, and *glnA* encodes glutamine synthetase. Plasmid pRL136 consists of the 1.85 kb *HindIII* fragment carrying *P_{nifHDK}* cloned into pRL119. Plasmid pRL562 consists of the 1.0 kb *HindIII*–*MspI* fragment carrying *P_{nifHDK}* cloned into pRL561. Plasmid pRL584 consists of the 2.4 kb *Sau3AI* fragment carrying *P_{rbcLS}* cloned into pRL579. Plasmid pRL559 consists of the 2.4 kb *HinPI*–*EcoRI* fragment carrying *P_{glnA}* cloned into pRL488. For details see Materials and methods.

Fig. 2. Light emission from strains of *Anabaena* PCC 7120 starved aerobically for nitrogen. (A) and (B) show *Anabaena* PCC 7120::pRL562 (*P_{nifHDK}*–*luxAB*) incubated 20 h in medium lacking combined nitrogen, bubbled with $\text{Ar}/\text{O}_2/\text{CO}_2$ (80:19:1). Total luciferase activity was 1100×10^7 quanta/ μg chlorophyll/min. (C) and (D) show *Anabaena* PCC 7120::pRL584 (*P_{rbcLS}*–*luxAB*) incubated for 1 day in medium lacking combined nitrogen, shaken under air. (A) and (C) are video images and (B) and (D) are images of luminescence captured and processed as described in Materials and methods. Light was collected 14 min and 10 min for (B) and (D) respectively. Bar = $10 \mu\text{m}$.

were used to ascertain the spatial pattern of promoter activity under conditions in which both oxygen and combined nitrogen had been removed from the culture. The pattern of luciferase activity in anaerobically starved filaments of *Anabaena* carrying $P_{nifHDK}-luxAB$ is shown in Figure 3A and B. In this image and in many others of filaments carrying $P_{nifHDK}-luxAB$, visibly differentiated cells invariably emitted light, but not all cells that emitted light could be distinguished from neighboring cells. Complete

Fig. 3. Light emission from strains of *Anabaena* starved anaerobically for nitrogen. (A) and (B) show *Anabaena* PCC 7120::pRL562 ($P_{nifHDK}-luxAB$) incubated 17 h in medium lacking combined nitrogen, bubbled with N_2/CO_2 (99:1). Total luciferase activity was 570×10^7 quanta/ μ g chlorophyll/min. (C) and (D) show *Anabaena* PCC 7120::pRL584 ($P_{rbcLS}-luxAB$) incubated 40 h under the same conditions. Total luciferase activity was 3000×10^7 quanta/ μ g chlorophyll/min. (E) and (F) show *Anabaena* PCC 7210 [pRL559] ($P_{glnA}-luxAB$) incubated for 24 h in medium lacking combined nitrogen, bubbled with Ar/CO_2 (99:1). Total luciferase activity was 1200×10^7 quanta/ μ g chlorophyll/min. The immature heterocyst at the left in (E) and (F) is slightly distorted, perhaps in response to shear forces. (A), (C) and (E) are video images and (B), (D) and (F) are images of luminescence captured and processed as described in Materials and methods. Light was collected for 16 min, 10 min and 21 min for (B), (D) and (F) respectively. Bar = 10 μ m.

differentiation of heterocysts requires the presence of oxygen (Rippka and Stanier, 1978) and it is sometimes difficult to identify partially differentiated heterocysts without procedures that kill the filaments (Neilsen *et al.*, 1971).

Numerous laboratories have performed anaerobic inductions of heterocyst differentiation by adding 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to a culture placed under an Ar/CO_2 atmosphere. DCMU blocks electron flow through photosystem II and the concomitant production of oxygen. Under our conditions, DCMU was not necessary for induction of *nifHDK* (see following section). The pattern of luciferase expression observed in a filament from an induced culture incubated with 10 μ M DCMU for 7 h was similar to that seen in Figure 3B, as was the pattern in an anaerobically induced culture of a *hetA* mutant, PS256-17 (Cai and Wolk, 1990), carrying the $P_{nifHDK}-luxAB$ fusion in the chromosome (data not shown). The *hetA* gene is required for normal assembly of the heterocyst envelope (Wolk *et al.*, 1988), which limits diffusion of oxygen into the mature heterocyst (Murry and Wolk, 1989).

Filaments of *Anabaena* carrying $P_{rbcLS}-luxAB$ were also observed after induction under anaerobic conditions (Figure 3C and D). All cells in the figure except the two heterocysts emitted light. Similar results were obtained whether the gas phase consisted of N_2/CO_2 or Ar/CO_2 . In addition, we observed the pattern of expression from a strain carrying $P_{glnA}-luxAB$. The *glnA* gene encodes glutamine synthetase, which is active in heterocysts and vegetative cells alike under aerobic conditions (Dharmawardene *et al.*, 1973). Luciferase activity in this strain was visualized in all cells of a filament (Figure 3E and F).

The relative light flux density from cells along the filament was quantitated by integrating c.p.m. over the area of each cell (Table I). From this analysis it appears that $P_{nifHDK}-luxAB$ expression is ~ 50 -fold higher in heterocysts than in vegetative cells (after subtraction of background counts). Conversely, $P_{rbcLS}-luxAB$ expression is ~ 7 -fold lower in heterocysts than in vegetative cells. These values must be interpreted with caution since light emission from vegetative cells (in the case of $P_{nifHDK}-luxAB$) and heterocysts (in the case of $P_{rbcLS}-luxAB$) is very close to electronic background. Expression of $P_{glnA}-luxAB$ is equal in the two types of cells within a factor of two.

Fig. 4. Quantitation of light emission across anaerobically induced filaments. (A) shows luciferase activity resulting from expression of $P_{nifHDK}-luxAB$ in a heterocyst (rightmost heterocyst in Figure 3A and B), and (B) shows luciferase activity resulting from expression of $P_{rbcLS}-luxAB$ in a vegetative cell (leftmost complete cell in Figure 3C and D). Each bar represents relative light flux density from a region ~ 0.5 μ m in width parallel to the axis of the filament. Dotted lines show the position of the cell boundaries. The solid bar at the side of each graph represents one standard deviation in each direction about the mean of the background counts, which is indicated by a line through the bar.

Emitted light was not always confined to the cell boundaries derived from the bright-field images (Figure 3). The spatial resolution obtainable by quantitating luciferase expression was assessed by counting light within regions taken progressively further from the axis of the filament (Figure 4). Counts fell rapidly with increasing distance and reached background levels outside of the cells within 1–2 μm from the cell boundaries. The patterns (Figure 5)

Fig. 5. Quantitation of light emission along anaerobically induced filaments. (A), (B) and (C) show luciferase activity resulting from expression of P_{nifHDK} – $luxAB$ (in Figure 3B), P_{rbcLS} – $luxAB$ (in Figure 3D) and P_{glnA} – $luxAB$ (in Figure 3F) respectively. Each bar represents relative light flux density from a region $\sim 0.5 \mu\text{m}$ in width within a single cell in one of the filaments shown in Figure 3. Dotted lines show the boundaries between cells. An asterisk above a group of intervals indicates that they comprise a heterocyst. The solid bar at the side of each graph represents one standard deviation in each direction about the mean of the background counts, which is indicated by a line through the bar.

obtained by counting light within regions taken along the filaments shown in Figure 3 suggest that light emission from P_{rbcLS} – $luxAB$ assigned to heterocysts in Table I is in fact attributable to spillover from adjacent cells and to imperfect alignment of the cell outline with the image of luminescence. If the bottom of the trough at the position of each heterocyst is taken as the measure of light emission from these cells, then expression of P_{rbcLS} – $luxAB$ in heterocysts is not significantly above electronic background. We do not know the significance, if any, of apparent regularities in the pattern of light emission of the filament carrying P_{rbcLS} – $luxAB$ (Figure 5B).

Formation of pattern and localization of P_{nifHDK} activity in mutant *Anabaena* PCC 7118

The time course of expression of $nifHDK$ was followed by means of a $nifHDK$ – $lacZ$ transcriptional fusion in *Anabaena* PCC 7118, a mutant in which heterocysts are not formed. Plasmid pRL136, carrying $lacZ$ fused to the 5' end of the $nifHDK$ operon (see Figure 1), was transferred into *Anabaena* PCC 7118. Figure 6 shows the time course of induction of β -galactosidase activity in the resulting strain. Induction of P_{nifHDK} – $lacZ$ first appears between 13 and 16 h after removal of combined nitrogen from the medium. The addition of DCMU resulted in a progressive decrease in the rate of induction of P_{nifHDK} – $lacZ$. The drug was therefore omitted in most experiments. β -Galactosidase activity remained at the uninduced level when nitrate was present and was similarly unaffected by starvation for sulfur (data not shown).

Induction of P_{nifHDK} – $lacZ$ in the mutant strain was accompanied by a diminution of average filament length (Figures 6 and 7). Fragmentation was not seen if the strain was incubated in the presence of nitrate (i.e. under non-inducing conditions). Sulfur starvation of *Anabaena* PCC 7118::pRL136 or nitrogen starvation of *Plectonema boryanum*, a cyanobacterium that does not differentiate, resulted in fragmentation only after several days of incubation (Figure 7). The rapid fragmentation experienced by nitrogen-starved *Anabaena* PCC 7118 cannot be explained by random breakage of the filaments. Random breakage would result in a filament size distribution that decreases monotonically as an exponential function.

[If every cell–cell junction were equally likely to rupture with a probability of p , then a fragment of length n would be less probable than a fragment smaller by one cell by a factor of $(1-p)$, the probability that the one extra

Table I. Comparison of light emission from vegetative cells and heterocysts

Strain	Fusion	Mean light flux density (counts per minute/ μm^2) ^a		
		Vegetative cells	Heterocysts	Background
Aerobic induction				
PCC 7120::pRL562	$P_{nifHDK}-luxAB$	0.30 ± 0.03 (18)	3.9 (2)	0.24 ± 0.02 (42)
PCC 7120::pRL584	$P_{rbcLS}-luxAB$	5.9 ± 0.5 (18)	0.96 (2)	0.25 ± 0.02 (44)
Anaerobic induction				
PCC 7120::pRL562	$P_{nifHDK}-luxAB$	0.30 ± 0.04 (18)	5.1 (2)	0.21 ± 0.02 (44)
PS256::pRL562	$P_{nifHDK}-luxAB$	0.31 ± 0.04 (19)	3.6 (2)	0.24 ± 0.02 (44)
PCC 7120::pRL584	$P_{rbcLS}-luxAB$	4.2 ± 0.3 (24)	0.83 (2)	0.30 ± 0.02 (47)
PCC 7120[pRL559]	$P_{glnA}-luxAB$	0.88 ± 0.07 (19)	0.56 (2)	0.22 ± 0.02 (42)

^aMean values were derived from unprocessed light flux densities from exposures that are presented in processed forms in Figures 2 and 3. Each number is followed by the standard deviation of the mean and by the number of measurements (in parentheses). Background values were derived as explained in Materials and methods.

junction has not broken. Therefore, the relative frequency of fragments having a given length (n) decreases monotonically with length and can be expressed as: $p(1-p)^{(n-1)}$.]

The mutant, however, exhibits a highly non-random filament size distribution (Figure 8A), with a peak centered at ~ 7 cells (and a lesser peak at ~ 15 cells). The late fragmentation observed with sulfur-starved *Anabaena* PCC 7118 and nitrogen-starved *P. boryanum* is nearly random (Figure 8B and C).

Heterocysts were not observed in *Anabaena* PCC 7118 under any incubation condition. However, unusual looking cells appeared in filaments after ~ 1 day of starvation for nitrogen under anaerobic conditions. These cells were darker and more granular than most cells and were often found at the end of filaments. When a filament contained more than one of these cells, at least several normal looking cells invariably intervened (Figure 9A). Some cells appeared to have an empty region, as if the cytoplasm had partially retracted from the cell envelope (Figure 9C).

In wild-type *Anabaena*, photosystem II is lost in heterocysts (Donze *et al.*, 1972), resulting in the loss of

Fig. 6. Induction of $P_{nifHDK-lacZ}$ in mutant *Anabaena* PCC 7118. *Anabaena* PCC 7118::pRL136 was incubated in medium lacking combined nitrogen, bubbled with Ar/CO_2 (99:1). At 16.5 h after the beginning of the incubation, DCMU was added in DMSO to one culture (open circles) to a final concentration of $10 \mu M$, while incubation of a duplicate culture (closed circles) continued without any addition. Samples were taken from the latter culture for counting cells per filament. The standard error of the mean for these measurements varied from 5 to 20% of the mean. β -Galactosidase activities are expressed as $OD_{420}/min/ng$ chlorophyll.

Fig. 7. Fragmentation of filaments after starvation. Cultures were incubated as in Figure 6. Closed circles, *Anabaena* PCC 7118::pRL136 in BG11₀ (no nitrate); open circles, *Anabaena* PCC 7118::pRL136 in BG11 (nitrate); open triangles, *Anabaena* PCC 7118::pRL136 in sulfate-deficient BG11; closed squares, *P. boryanum* in BG11₀. Each measurement represents a minimum of 770 cells counted.

phycocyanin-dependent fluorescence (van Gorkom and Donze, 1971). Loss of fluorescence in a pattern reminiscent of heterocyst spacing can be seen in mutant *Anabaena* PCC 7118 starved for nitrogen under anaerobic conditions. The unusual cells described above do not fluoresce (Figure 9B and D), but cells could be observed that were non-fluorescent

Fig. 8. Length profile of filaments after starvation. Distributions of filament lengths in samples from the experiment shown in Figure 7. Samples were selected that had nearly identical mean filament lengths. Similar results were obtained when the median was used as the selection criterion. Distributions are presented as rolling averages, with a window of two cells per filament to dampen a bias towards even numbers of cells per filament seen in many samples. The insets show the same data plotted in semilogarithmic coordinates. The dashed line represents the best fitting exponential curve. The value of P given for each set of data represents the probability (taken from χ^2 analysis) that the set is consistent with the best fitting curve. The rightmost 5% of the theoretical curve was not considered in this analysis. The value of N represents the number of filaments counted. (A) *Anabaena* PCC 7118::pRL136 in BG11₀, 23.5 h after induction. $N = 293$, mean = 10.8 cells/filament, $P = 6 \times 10^{-11}$. (B) *Anabaena* PCC 7118::pRL136 in sulfate-deficient BG11, 112 h after induction. $N = 297$ (1 off scale), mean = 11.2 cells/filament, $P = 0.26$. (C) *P. boryanum* in BG11₀, 134 h after induction. $N = 200$ (7 off scale), mean = 10.9 cells/filament, $P = 0.67$.

but did not appear otherwise unusual. *Anabaena* PCC 7118 induced under air also displayed a pattern of non-fluorescent cells (Figure 10A). Under these conditions as well, cells that did not fluoresce often appeared granular (Figure 10B and C), but empty regions were never observed. Transparent cells that appeared lysed could be seen under aerobic or anaerobic conditions, at termini or internal positions in which a granular cell might otherwise be expected (Figure 10D and E). Non-random fragmentation occurred under aerobic conditions with a similar time course as under anaerobic conditions, but expression of $P_{nifHDK-lacZ}$ was quite low (data not shown).

A $P_{nifHDK-luxAB}$ fusion was introduced into *Anabaena* PCC 7118 in order to ascertain where nitrogenase genes are expressed. Figure 11 shows a filament of the resulting strain induced under anaerobic conditions. The two centers of light emission had light flux densities (unprocessed) of 1.94 and 2.69 c.p.m./ μm^2 . The average of the light flux densities (unprocessed) of the remaining cells of the filament was 0.32 ± 0.03 c.p.m./ μm^2 . One of the two centers is a cell of the type described above, in which the cytoplasm appears

to be pulling away from the cell envelope. Light-emitting granular cells were also observed (data not shown).

Discussion

Two paradigms have greatly influenced recent research into the regulation of nitrogenase expression and patterned heterocyst differentiation in the cyanobacterium *Anabaena*:

Fig. 9. Filaments of *Anabaena* PCC 7118 starved for nitrogen under anaerobic conditions. *Anabaena* PCC 7118::pRL136 ($P_{nifHDK-lacZ}$) incubated for 23 h in medium lacking combined nitrogen, bubbled with Ar/CO_2 (99:1). (B) and (D) are fluorescence images of the filaments in (A) and (C) respectively, excited with light centered around 546 nm with a bandwidth of 6 nm. (A) and (B) show a filament with spaced, granular cells. (C) and (D) show a filament in which a non-fluorescent cell appears to have an empty region. Emission, isolated with a filter that blocked wavelengths <590 nm, appeared red. Bar = 10 μm .

Fig. 10. Filaments of *Anabaena* PCC 7118 starved for nitrogen under aerobic conditions. *Anabaena* PCC 7118::pRL136 ($P_{nifHDK-lacZ}$) incubated in medium lacking combined nitrogen, shaken under air. (A), (C) and (E) are fluorescence images, obtained as described in Figure 9. (A) shows the long-range pattern of non-fluorescent cells within a filament. (C) and its corresponding bright-field image, (B), show a spaced pattern of granular, non-fluorescent cells. (E) and its corresponding bright-field image, (D), show an apparently lysed cell midway between two non-fluorescent cells. (A), (D) and (E) are of samples taken at 25 h, and (B) and (C) are of a sample taken at 23 h. Bar = 50 μm in (A), 10 μm in (B–E).

Fig. 11. Light emission from strain of *Anabaena* PCC 7118 carrying $P_{nifHDK-luxAB}$ fusion. (A) and (B) show *Anabaena* PCC 7118::pRL562 ($P_{nifHDK-luxAB}$) incubated for 38 h in medium lacking combined nitrogen, bubbled with N_2/CO_2 (99:1). Total luciferase activity was 300×10^7 quanta/ μg chlorophyll/min. (A) is a video image, and (B) is a luminescence image captured and processed as described in Materials and methods. Light was collected 9 min for (B). Bar = 10 μm .

transcriptional control over differentiation, as seen in *B. subtilis* (Losick *et al.*, 1988), and environmental control over *nifHDK* transcription, as seen in *K. pneumoniae* (Gussin *et al.*, 1986). When these paradigms are applied to *Anabaena* simultaneously (Haselkorn *et al.*, 1983), nitrogenase expression and heterocyst differentiation can be viewed as separately regulated, the *nif* genes being activated by the low levels of oxygen and combined nitrogen presumed to exist in the mature heterocyst. The work presented here favors the alternative view that the induction of *nif* genes is under developmental control, responding to one or more signals that also direct heterocyst differentiation.

Developmental versus environmental control over

P_{nifHDK}

If the *nif* genes were regulated in direct response to cellular levels of oxygen and nitrogen, then removing both from the medium should lead to expression in all cells. However, the expression of *P_{nifHDK}* fused to *luxAB* (encoding luciferase) was largely confined to differentiated cells, even under conditions of anaerobiosis (Figures 2A and B and 3A and B). Expression appeared to be ~50-fold higher per cell in heterocysts than in vegetative cells (Table I). This ratio must be considered a crude approximation and probably an underestimate, since the measured values for luciferase activity in vegetative cells were very close to the levels of luciferase-independent noise, and luciferase activity in heterocysts is partially limited by substrate (J.Elhai and C.P.Wolk, unpublished results).

To assess the possibility that heterocysts intrinsically support a higher level of expression of *luxAB*, that operon was placed under the control of promoters expected to direct expression in a pattern different from *P_{nifHDK}*. *P_{rbcLS}*–*luxAB* showed a pattern of expression opposite to that of *P_{nifHDK}*–*luxAB* (Figures 2C and D and 3C and D, and Table I), consistent with the confinement of ribulose biphosphate carboxylase activity to vegetative cells (Winkenbach and Wolk, 1973; Codd and Stewart, 1977) and the virtual absence of *rbcLS* message from isolated heterocysts (Golden *et al.*, 1988). Filaments induced anaerobically were reported to lack *rbcLS* message (Haselkorn *et al.*, 1983), but since under similar conditions there is appreciable *P_{rbcLS}*–*luxAB* expression in vegetative cells, the possibility should be considered that the stability of *rbcLS* message is decreased by starvation for nitrogen. Glutamine synthetase specific activity is marginally greater in heterocysts than in vegetative cells (Dharmawardene *et al.*, 1973), and the *P_{glnA}*–*luxAB* fusion indicated comparable promoter activity in both cell types (Figure 3E and F and Table I). These and similar results indicate that under the conditions of the above experiments, luciferase activity in cells carrying *P_{nifHDK}*–*luxAB* reflected primarily the regulation of the promoter driving its expression rather than intrinsic limitations in heterocysts or vegetative cells.

It is also unlikely that oxygen present during the experiment was so high as to suppress *P_{nifHDK}*–*luxAB* expression selectively in vegetative cells. DCMU, an inhibitor of photosynthesis, is often added to the incubation medium in anaerobic experiments to prevent the accumulation of oxygen. However, DCMU significantly reduced expression of *P_{nifHDK}*–*lacZ* (Figure 5) and *P_{nifHDK}*–*luxAB* (data not shown) and also prevents full induction of nitrogenase activity (Murry *et al.*, 1984). By continuously bubbling gas through a dilute culture in the absence of

DCMU, we achieved stable induction of *P_{nifHDK}* in both *Anabaena* PCC 7120 and *Anabaena* PCC 7118, which has little *P_{nifHDK}* activity under air (Figure 6 and J.Elhai and C.P.Wolk, unpublished results). Even in the presence of DCMU, the localization of *P_{nifHDK}*–*luxAB* to heterocysts was evident, although light emission was much weaker. Anaerobically grown filaments were exposed to oxygen during the manipulations required to obtain images of luminescence, but luciferase activity decreased relatively little during this period. We believe, therefore, that the oxygen levels during incubation were sufficiently low and the subsequent manipulations sufficiently rapid that the observed pattern of luminescence was not affected by oxygen.

We conclude that *P_{nifHDK}* is active only in differentiated cells of *Anabaena* PCC 7120 even when the culture is induced anaerobically. This result implies that if *P_{nifHDK}* is under environmental control, then the promoter is regulated by a condition besides anaerobiosis that differs between differentiated and non-differentiated cells, for example, a greater degree of nitrogen deprivation in differentiated cells. Alternatively, *P_{nifHDK}* may require for activity a factor that appears only in differentiated cells. This possibility is supported by the finding that *P_{hetA}*, which exhibits a similar spatial pattern of activity to *P_{nifHDK}*, is induced under aerobic or anaerobic conditions several hours earlier than *P_{nifHDK}* (Holland and Wolk, 1990; Holland, Elhai and Wolk, unpublished data). Developmental control of *P_{nifHDK}* does not preclude the possibility that levels of oxygen and combined nitrogen also regulate transcription from this promoter. Indeed, admission of air into a culture that was induced under anaerobic conditions leads to a rapid loss of *nifHDK* message (Haselkorn *et al.*, 1983; Helber *et al.*, 1988).

A component of nitrogenase has been localized immunologically to heterocysts of a free-living strain of *Anabaena* induced under anaerobic conditions (Murry *et al.*, 1984) and of a symbiotic cyanobacterium living heterotrophically under microaerobic conditions (Bergman *et al.*, 1986). These findings support the view presented here that a developmental signal is required for transcription of *nif* genes. Two groups (van Gorkom and Donze, 1971; Rippka and Stanier, 1978) reached the contrary conclusion, after failing to find gradients of nitrogenous polymers emanating from heterocysts once N₂ was readmitted to cultures induced in the absence of both N₂ and O₂. Gradients would be expected, however, only if fixed nitrogen were limiting in the presence of N₂, and it is very likely that fixed nitrogen was not limiting in those experiments. The high levels of nitrogenase activity induced in the absence of N₂ slowly fall upon readmission of the gas (Neilson *et al.*, 1971), suggesting that the enzyme capacity is initially higher than that needed to satisfy the nitrogen requirement of the filaments.

A line of reasoning that points independently to the interpretation that nitrogenase activity is spatially heterogeneous stems from the observation that an 11 kb element is excised from the *nifD* gene during heterocyst maturation (Golden *et al.*, 1985). Under aerobic conditions, the small fraction of the DNA that is rearranged is present primarily if not exclusively in heterocysts (Golden *et al.*, 1985) and excision of this element is absolutely required for nitrogenase activity (Golden and Wiest, 1988). The same rearrangement occurs in anaerobically induced *Anabaena* PCC 7118 and to roughly the same extent (Martin Mulligan, personal communication). If the rearranged chromosomes are

heterogeneously distributed under anaerobic conditions, as they are under aerobic conditions, then only a minor fraction of anaerobically induced cells would have an intact *nifD* gene, and only that fraction could produce active nitrogenase.

Differentiation of mutant *Anabaena* PCC 7118 in response to nitrogen deprivation

Perhaps the strongest argument favoring purely environmental control over *nif* gene expression rests on the existence of a mutant strain, *Anabaena* PCC 7118, that cannot make heterocysts but can fix nitrogen if incubated in the absence of oxygen and combined nitrogen (Rippka and Stanier, 1978). Mutant *Anabaena* PCC 7118 appears to be virtually identical to *Anabaena* PCC 7120 on the basis of morphology (Rippka, 1988), DNA reassociation (Lachance, 1981) and restriction pattern in the regions of *rbcLS* and *nifHDK* (J.Elhai and C.P.Wolk, unpublished results). Several lines of evidence (see below) indicate that *Anabaena* PCC 7118, like the wild-type strain, does develop a pattern in response to nitrogen deprivation, although one that is not obvious by bright-field microscopy, and P_{nifHDK} is expressed within only certain cells in the filament. Anaerobic nitrogen fixation by this mutant strain [and, we suggest, similar strains (Spence and Stewart, 1987; Helber *et al.*, 1988)] does not, therefore, favor the idea of environmental control over *nif* gene expression.

Incubation of the mutant in the absence of combined nitrogen results in a non-random fragmentation of the filaments, which cannot be ascribed to starvation *per se* (Figures 6, 7 and 8). A pattern of well spaced, non-fluorescent cells is seen in the mutant upon nitrogen starvation (Figures 9 and 10). These cells are often darker and more granular than their neighbors, and occasionally contain regions in which the cytoplasm appears to have retracted from the cell wall (Figure 9C). Granular cells and cells containing empty regions are sites of P_{nifHDK} activity (Figure 11 and data not shown). It is tempting to explain these observations in terms of aborted heterocyst differentiation. The distribution of fragment lengths is reminiscent of the spacing between heterocysts in wild-type strains (Wolk and Quine, 1975). Loss of absorption (Thomas, 1972) and fluorescence by phycocyanin (Donze, 1973) occurs early in heterocyst differentiation in wild-type *Anabaena*. Retraction of the cytoplasm from the cell wall may be related to the ease with which the envelopes of immature and mature heterocysts but not of vegetative cells are detached from the cell protoplast by acid (Neilson *et al.*, 1971). The pattern of expression of P_{nifHDK} -*luxAB* in *Anabaena* PCC 7118 supports the idea that pattern formation in this strain corresponds to cryptic heterocyst differentiation.

Luciferase is a useful tool to monitor the spatial distribution of gene activity

Because one product of the luciferase reaction is light, the activity of the enzyme can be assayed with exquisite sensitivity by means of a non-destructive assay. Luciferase may therefore be used to localize processes *in vivo*. Genes encoding luciferase from marine bacteria have been cloned and introduced into bacteria to follow macroscopic processes (Shaw and Kado, 1986; O'Kane *et al.*, 1988; Schauer *et al.*, 1988). Bacterial and firefly luciferase have also been used as transcriptional reporters in eukaryotic cells (Gallie *et al.*, 1989; Kirchner *et al.*, 1989). We have demonstrated that luciferase activity may be used to localize

the site of gene expression within single bacterial cells to a resolution of $\sim 1 \mu\text{m}$ (Figures 4 and 5). Quantitation is hampered by several problems, however, most notably the difficulty of putting all cells within a filament in focus and avoiding movement of the cells during long exposure times. Interpretation is also complicated if a specific activity is desired. We normalized activity to cross-sectional area, which is readily measured, but light is presumably emitted from the entire transected volume. The sharp decline in apparent activity near the edge of cells (Figures 4 and 5) is probably due to the error introduced by normalizing activity to area rather than volume. For slices of the size used, normalizing to area would underestimate activity by a factor of ~ 10 at the poles of a spherical cell. Despite these difficulties, differences in luciferase activity between adjacent cells are easily distinguishable. The technique may also be useful to monitor activities within specific compartments of living eukaryotic cells or of differentiating cells of *Bacillus*.

Materials and methods

Strains and plasmids

Anabaena PCC 7118 was obtained from Rosmarie Rippka, *Anabaena* PCC 7120 was obtained from Robert Haselkorn, and *P. boryanum* strain 594 was obtained from Shree Kumar Apte. *Anabaena* PS256-17 (Cai and Wolk, 1990) is a derivative of *Anabaena* PCC 7120 in which the *hetA* gene has been insertionally inactivated. Cyanobacterial strains were grown in modified BG11 (with nitrate) or modified BH11₀ (no combined nitrogen), made according to Rippka *et al.* (1979) except that ferric ammonium citrate was replaced by an equal weight of ferric citrate. When sulfate-deficient BG11 was used, MgSO_4 was replaced with an equal molar quantity of MgCl_2 . The strains were grown at 30°C under fluorescent lights ($\sim 60 \mu\text{Einsteins/m}^2/\text{s}$) on a rotary shaker. Neomycin-resistant strains were propagated in liquid media supplemented with 3 $\mu\text{g/ml}$ neomycin.

Plasmid pRL119 (Figure 1) is a vector designed to facilitate transcriptional fusions to *lacZ*. It was constructed by placing *trp-lacZ* on a 3.9 kb *Bam*HI-*Dra*I fragment from pLRK21 (Kroos and Kaiser, 1984) between the *Bam*HI and *Dra*I sites of pRL19B (Elhai and Wolk, 1988a). The *trp-lacZ* fusion is derived ultimately from pMC903 (Casadaban *et al.*, 1980). DNA was inserted into pRL119, using the following sites of its polylinker (asterisked sites occur nowhere else in the plasmid): *Hind*III*-*Cl*aI-*Hind*III*-*Xba*I*-*Bgl*II*-*Pst*I-*Bam*HI* (5' end of *lacZ*). There are translational stops in all three reading frames between the polylinker and the 5' end of *lacZ*.

Plasmid pRL488 (Figure 1) is a vector designed to facilitate transcriptional fusions to *luxAB*, on a plasmid that can replicate in both *Escherichia coli* and many strains of filamentous cyanobacteria. It is derived from pRL25 (Wolk *et al.*, 1988), with *luxAB* inserted at the unique *Bam*HI site of pRL25. The genes encoding luciferase from *Vibrio fischeri* were taken from pJE205 (Engelbrecht *et al.*, 1983) on a 2407 bp *Sst*I-*Dra*I fragment (Foran and Brown, 1988). This region is preceded by a polylinker taken from pUC19: *Pst*I-*Sal*I*-*Xba*I-*Bam*HI-*Sma*I*-*Kpn*I*-*Sst*I*-(5' end of *luxA*). There are translational stops in all three reading frames between the polylinker and the 5' end of *luxA*. The polylinker is preceded by a region corresponding to the 3' end of the major transcript of *cpcBA* of the cyanobacterium *Synechococcus* PCC 7002. The region was taken from pAQPR1 (de Lorimier *et al.*, 1984) as a 269 bp *Nco*I-*Xho*I fragment overlapping the 3' end of *cpcA* (Pilot and Fox, 1984).

Plasmids pRL561 and pRL579 (Figure 1) are vectors designed to facilitate transcriptional fusions to *luxAB*, on a plasmid that cannot replicate in cyanobacteria. They were constructed from pRL488 by deleting the cyanobacterial replicon and replacing the kanamycin resistance determinant with that from pRL447 (Elhai and Wolk, 1988a), in which the neomycin phosphotransferase gene is preceded by the strong promoter from *psbA* of *Amaranthus hybridus*. Plasmid pRL561 carries the polylinker: *Xba*I*-*Sal*I*-*Pst*I-*Sal*I*-*Xba*I*-*Bam*HI-*Sma*I*-*Kpn*I*-*Sst*I*. Plasmid pRL579 carries the polylinker [derived in part from pLC19H (Marsh *et al.*, 1984)]: *Xba*I*-*Sal*I*-*Bam*HI-*Sma*I*-*Eco*RI*-*Cl*aI*-*Eco*RV*-*(Xba*I*)-*Bgl*II*-*Xho*I*-*Sst*I*. The site in parentheses can be cut only if DNA is isolated from a strain lacking the Dam methylase.

Plasmid pRL136 carries P_{nifHDK} transcriptionally fused to *lacZ*. An 1849 bp *Hind*III-*Hind*III fragment containing P_{nifHDK} , *nifH* and the 5' end of *nifD* was taken from pAn154 (Mevarech *et al.*, 1980) and inserted between

the *Hind*III sites of pRL119, with the promoter pointing towards *lacZ*. Plasmid pRL562, carrying *P_{nifHDK}* transcriptionally fused to *luxAB*, was constructed by inserting a 1015 bp *Hind*III–*Msp*I fragment (Mevarech *et al.*, 1980; Mulligan and Haselkorn, 1989), containing *P_{nif}* and the 5' end of *nifH* derived from pAn154, between *Pst*I and *Acc*I sites within the polylinker of pRL561, with the promoter pointing towards *luxAB*. The *Hind*III and *Pst*I sites were joined by means of a 38 bp *Hind*III–*Pst*I fragment from the polylinker of pRL119.

Plasmid pRL584 carries *P_{rbcLS}* transcriptionally fused to *luxAB*. A 2.4 kb *Sau*3AI–*Sau*3AI fragment containing *P_{rbcLS}* and the 5' end of *rbcL* was taken from pAn601, a 7.3 kb subclone of pAn600 (Curtis and Haselkorn, 1983; Stephanie Curtis, personal communication) and inserted in the *Bgl*II site of pRL579. Plasmid pRL559 carries *P_{glnA}* transcriptionally fused to *luxAB*. A 394 bp *Hin*PI–*Eco*RI fragment (Tumer *et al.*, 1983) containing *P_{glnA}* and the 5' end of *glnA* was taken from pAn503 (Fisher *et al.*, 1981) and inserted between the *Sal*I and *Sst*I sites of pRL488, with the promoter(s) pointing towards *luxAB*. The *Hin*PI and *Sal*I sites were joined by means of a 14 bp *Cl*aI–*Xho*I fragment from pIC20H (Marsh *et al.*, 1984), and the *Eco*RI and *Sst*I sites were joined by means of a 6 bp fragment from the same plasmid. The 394 bp fragment contains all four transcriptional start sites previously identified (Tumer *et al.*, 1983). It is possible, however, that the small fragment bearing *P_{glnA}* used in this study lacks upstream sequences that are required for proper regulation.

Strains of *Anabaena* carrying gene fusions were constructed by transferring the appropriate plasmid into the strain by conjugation from donors carrying the helper plasmid pRL528 (Elhai and Wolk, 1988b). Plasmids based on pRL119, pRL561 and pRL579 cannot replicate in *Anabaena*, but their derivatives pRL136, pRL562 and pRL584, all of which carry cloned *Anabaena* DNA, readily integrate into the chromosome by single recombination, yielding a duplication of the cloned sequence.

Assays

Chlorophyll concentration was measured in a methanolic extract, using a factor of 13.4 to convert OD₆₆₅ to µg chlorophyll/ml (Mackinney, 1941). β-Galactosidase activity was measured essentially as described by Miller (1972), after cells were permeabilized by treatment with chloroform and SDS. Luciferase activity was measured in an ATP photometer (Lab-Line) by a procedure modified from that previously described (Schmetterer *et al.*, 1986). The reaction was initiated by adding to cyanobacteria suspended in 2 ml growth medium 200 µl of a 0.1% (v/v) emulsion of *n*-decanal in a 20 mg/ml solution of bovine serum albumin (Sigma, <0.005% fatty acids) in water. The emulsion was prepared by cavitating the mixture in an acid-washed glass tube until a faint blue translucence could be seen. The emulsion was allowed to age overnight before use, a procedure which increased the duration of luciferase activity.

Induction of gene expression after removal of nitrogen

Cultures were subcultured in BG11 several times prior to induction experiments. This was necessary to reduce the number of heterocysts that appear in cultures recently diluted from stationary phase [a phenomenon also reported with this strain by Meeks *et al.* (1983)]. Cultures were maintained very dilute: at the start of experiments, chlorophyll concentrations ranged from 0.1 to 0.7 µg/ml. Under these conditions, heterocysts were virtually absent. Nitrate was removed from cultures by several washes with BG11₀ until the calculated concentration of nitrate was no more than 2 µM. Cells were resuspended to the original concentration in acid-washed flasks containing BG11₀ supplemented with 5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 8.0, and 1 mM Na₂CO₃. The flasks were sealed with serum stoppers and bubbled with the appropriate gas mixture. Under these conditions, the pH of the culture was ~7.8. Contaminating oxygen was removed from N₂ (Umbreit, 1973) when that gas was used in anaerobic experiments. DCMU, 10 µmol/ml dimethyl-sulfoxide, was added to some cultures to a final concentration of 10 µM.

Microscopy and localization of luciferase activity

Fluorescence and phase contrast microscopy was performed with a Zeiss Axiophot microscope. Fluorescence was observed using a filter set, designed for rhodamine fluorescence, which transmits a 540–552 nm band of exciting light and blocks emission below 590 nm. Luminescence and video images were captured with a Hamamatsu Photonics System, model C1966-20 (Wick, 1987), cooled with a Peltier ring and attached to a Zeiss Universal microscope. The temperature of the microscope stage was maintained between 18°C and 23°C. Video images were photographed after subtraction of fixed background. Luminescence images (a representation of the photons counted at each pixel during the exposure) were processed further, using locally written software. The first goal of processing was to reduce electrical noise and to reverse the amplification of noise that results from charge spreading (Wick, 1987), which is inherent in the system. Each photon or

spurious count is amplified, on average, to a total of five counts that are distributed characteristically to adjacent pixels in alternate rows. To correct for charge spreading, the photon value for each pixel was replaced by the geometric mean of the original value and the average photon value within odd-numbered rows of a 7 × 7 array centered on the pixel. The row of the pixel was defined as zero. The graphs shown in Figures 4 and 5 and data shown in Table I were derived from unprocessed images of luminescence. The second goal was to associate luminescence with specific cells or regions. The outline of the cells was extracted by subjecting the video image to histogram equalization (Gonzalez and Wintz, 1987) and passing the transformed image through a threshold filter. Levels of electronically induced background counts were estimated by translating the outlines of the filaments and of the regions contained within them by approximately two cell widths to positions on either side of the filament.

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