JOURNAL OF BACTERIOLOGY, Apr. 2003, p. 2306–2314 Vol. 185, No. 7
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.7.2306–2314.2003
Copyright © 2003, American Society for Microbiology. All Rights Reserved.

Heterocyst-Specific Expression of *patB*, a Gene Required for Nitrogen Fixation in *Anabaena* sp. Strain PCC 7120

Kathryn M. Jones,† William J. Buikema, and Robert Haselkorn*

Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois 60637

Received 5 July 2002/Accepted 8 January 2003

The *patB* gene product is required for growth and survival of the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 in the absence of combined nitrogen. A *patB*::*gfp* fusion demonstrated that this gene is expressed exclusively in heterocysts. *patB* mutants have a normal initial pattern of heterocyst spacing along the filament but differentiate excess heterocysts after several days in the absence of combined nitrogen. Expression of *hetR* and *patS*, two critical regulators of the heterocyst development cascade, are normal for *patB* mutants, indicating that *patB* acts downstream of them in the differentiation pathway. A *patB* deletion mutant suffers an almost complete cessation of growth and nitrogen fixation within 24 h of combined nitrogen removal. In contrast, a new PatB mutant that is defective in its N-terminal ferredoxin domain, or a previously described mutant that has a frameshift removing its C-terminal helix-turn-helix domain, grows very slowly and differentiates multiple contiguous heterocysts under nitrogen-deficient conditions.

Anabaena sp. strain PCC 7120 is a filamentous cyanobacterium capable of both nitrogen fixation and oxygenic photosynthesis. When deprived of combined nitrogen, approximately every 10th cell along the filament differentiates into a nitrogen-fixing heterocyst. Heterocysts provide the anaerobic environment in which nitrogenase can function. The oxygen-evolving photosystem II complex is inactivated in these cells, and a semipermeable barrier to gases is provided by the heterocyst envelope, which consists of an inner glycolipid layer and an outer polysaccharide layer (15). The morphological and biochemical changes that take place during heterocyst differentiation provide the enzyme nitrogenase with an anaerobic environment and the large supply of reductant and ATP that it requires.

Many mutant strains of *Anabaena* sp. strain PCC 7120 exhibit an altered heterocyst spacing pattern. One of these, the Pat-2 strain, has a normal initial heterocyst pattern but accumulates multiple contiguous heterocysts after several days in the absence of combined nitrogen (N_o) (14). The mutant strain has a single-base deletion at position 1342 of the *patB* gene, resulting in loss of the C-terminal portion of the protein, including an HTH-3/HTH-XRE family DNA-binding motif. The N-terminal domain of the protein contains two putative 4Fe-4S centers with a high degree of similarity to bacterial-type ferredoxin II proteins.

The Pat-2 strain was isolated in a screen for mutants that grow poorly in the absence of combined nitrogen. Consistent with the requirement for functional PatB under nitrogen-limiting conditions, the *patB* message was found to be strongly induced in the wild-type strain around 12 h after removal of nitrogen. The *patB* message continues to accumulate after the initial induction, although it never reaches a very high level (14). In this work, we have determined that *patB* is expressed specifically in heterocysts and that the heterocyst development regulators *hetR* and *patS* are expressed normally in *patB* mutants through the first round of heterocyst differentiation. We have also constructed two new mutants in *patB* to further explore its function. One of these carries a deletion of the entire *patB* gene. The other carries six Cys→Ala mutations predicted to disrupt the PatB Fe-S centers.

MATERIALS AND METHODS

Cloning and plasmid construction. Cloning methods are described elsewhere (11, 19). All sequencing reactions were performed by the University of Chicago Cancer Research Center DNA Sequencing Facility.

The transcriptional reporter *patB*::*gfp* was constructed by PCR amplification of 1.0 kb upstream of the *patB* start codon using primers that were designed with, respectively, an *Xba*I site and a *Sma*I site at their 5′ ends. The PCR product was digested with these enzymes, and the *Xba*I site was filled with T4 DNA polymerase. The resulting blunt-ended fragment was cloned into the pAM1956 *Sma*I site directly upstream of *gfp* mut2, which was kindly provided by H. S. Yoon and J. W. Golden (Texas A&M University) (22). pAM1956 carries a neomycin-kanamycin resistance marker (Nm^r/Km^r). The *patS*::*gfp* reporter was also provided by H. S. Yoon and J. W. Golden (22). A *hetR*::*gfp* reporter was described previously (4).

The *patB* open reading frame (ORF) deletion constructs p*patB*ΔΩRL271 and p*patB*ΔΩRL278 were made by PCR amplification of a 1.0-kb sequence upstream of *patB* and of a 1.0-kb segment downstream of *patB*. The upstream product was digested with *Xba*I and *Sma*I, and the downstream product was digested with *Sma*I and *Pst*I, and both fragments were ligated into *Xba*I/*Pst*I-digested BluescriptKS (Stratagene). The resulting construct was digested with *Sma*I and then ligated with a *Sma*I-digested streptomycin-spectinomycin resistance cassette (Sm^r/Sp^r) (9). A *Xho*I/*Sac*I fragment containing this whole construct was moved from BluescriptKS to the *sacB*-containing suicide vectors pRL271 or pRL278 for conjugation into *Anabaena* sp. strain PCC 7120.

The *patB* ORF Fe-S center mutagenesis construct, pFeS*patB*replace, was made by first cloning the *patB* ORF into the pAlter-EX2 (Promega) vector. Cysteines 1 to 4 and 7 and 8 of the *patB* FeS centers were changed to alanines using mutagenic primers. The mutagenized *patB* ORF was cloned into p*patB*ΔΩRL278 (see above) at a *Bam*HI site left behind by deletion of the Ω cassette. A 0.3-kb *Sfu*I/*Xho*I fragment had also been deleted from this plasmid. The construct pwt*patB*replace was made by ligating the unmutagenized *patB* ORF into this modified p*patB*ΔΩRL278. The resulting constructs have the *patB* ORF separated from their native flanking sequences by a *Bam*HI site and a *Sma*I half-site.

* Corresponding author. Mailing address: Department of Molecular Genetics and Cell Biology, University of Chicago, 920 East 58th St., Chicago, IL 60637. Phone: (773) 702-1069. Fax: (773) 702-2853. E-mail: r-haselkorn@uchicago.edu.

† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

The *patB* expression plasmid p*petE*::*patB* contains the copper-inducible *petE* promoter fused to the complete *patB* ORF and is described in detail in reference 10.

Transformation and conjugation are described in detail in references 10, 14, and 19.

Cell culture. *Escherichia coli* cultures were maintained by standard methods (19). *Anabaena* sp. strain PCC 7120 culture methods were as described previously (11, 18). Large cultures for RNA isolation, or cultures which required a high level of gassing, were grown in stirred bottles illuminated by cool white fluorescent bulbs at 30 to 40 $\mu E/m^2/s$ and gassed with a 2 to 3% CO_2/air mixture. The pH of gassed cultures was maintained with 25 or 50 mM HEPES, pH 8.0. Culture maintenance and transfer from nitrogen-replete medium to N_o medium were as described previously (10). Cell growth was assessed by cell counting or by measuring the absorbance at 750 nm (20, 21).

Complementation of mutations in the *patB* ORF. The ability of the *patB* ORF to complement *patB* mutants was tested by conjugating either the p*petE*::*patB* copper-regulated *patB*-expression plasmid or the control p*petE* plasmid into the mutant of interest (10). Exconjugants were selected on BG11-NO_3 plates containing 30 μg of neomycin sulfate/ml and in the absence of added $CuSO_4$. Isolates were then tested for their ability to grow on BG11 N_o, 30-μg/ml neomycin sulfate plates in the absence of added $CuSO_4$. The trace amounts of copper derived from the glassware were sufficient to induce very low-level *patB-cat* hybrid message expression (data not shown). This level of expression was sufficient to complement all *patB* mutants tested.

Microscopy. Cells were pipetted onto 1% agar cushions containing BG11 for photography. The strain carrying the *patB*::*gfp* construct was photographed with 400 ASA film in a Contax 167MT camera attached to a Zeiss Axioskop microscope. Images of green fluorescent protein (GFP) fluorescence were recorded by illuminating with 450- to 490-nm light from a Zeiss HBO100W/2 source and photographing emission through a 510-nm narrow-band-pass filter with a 16-s exposure. Red emission from chlorophyll was photographed without the 510-nm filter. Images of the strains carrying the *patS*::*gfp* reporter were captured using an Axiovision color charge-coupled device camera attached to a Zeiss Axioplan2 microscope. The CCD images of GFP fluorescence were acquired when the GFP was excited at 450 to 490 nm and viewed through the 500- to 550-nm filter of the Endow GFP filter set (Chroma).

Nitrogenase activity assays. Reduction of acetylene to ethylene was measured by gas chromatography as described previously (7, 10).

Northern blotting and hybridization. At selected times following nitrogen step-down, 300 ml was removed from a 4-liter culture and RNA was isolated using the Ambion Totally RNA kit protocol with modifications described elsewhere (10). RNA loading on formaldehyde-agarose gels was assessed by scanning baked blots with the blue laser of the Storm 860 phosphorimager at a photomultiplier voltage of 900. Under these conditions the imager detects ethidium bromide-stained rRNA bands (8). The doublet 23S rRNA bands of *Anabaena* sp. strain PCC 7120 were quantified and used to normalize the ^{32}P signal from probes to specific mRNAs. Probes were labeled by random priming with the Ambion Strip-Easy DNA probe synthesis kit or the Roche Random primed DNA labeling kit, using [^{32}P]dCTP or [^{32}P]dATP (Amersham). Blots were imaged by exposure to a Type BAS-III Fuji phosphorimager screen for 2 h. The exposed screen was scanned with a Molecular Dynamics Storm 860 PhosphorImager and analyzed with Molecular Dynamics IQMac software.

RESULTS

patB::*gfp* is expressed exclusively in heterocysts, in the middle to late stages of heterocyst development. *patB* transcripts accumulate in cultures grown in the absence of combined nitrogen (14). Analysis of filaments carrying a *patB*::*gfp* transcriptional fusion shows that this expression occurs solely in heterocysts (Fig. 1A). Wild-type filaments carrying the *patB*::*gfp* fusion have no GFP fluorescence when grown with either $NaNO_3$ or $(NH_4)_2SO_4$ as a nitrogen source, but beginning at 16 h after transfer to N_o medium, fluorescence is visible in cells spaced at 10-cell intervals. This fluorescence increases in intensity for the next 2 days. Heterocysts can be distinguished by the loss of chlorophyll fluorescence (Fig. 1B) due to the breakdown of photosystem II. No GFP fluorescence was observed from the *patB*::*gfp* reporter strain earlier than 16 h when heterocyst differentiation was synchronized by growth in 1 mM $(NH_4)_2SO_4$ prior to transfer to N_o medium. These data agree with the *patB* expression profile observed on Northern blots (Fig. 1C). The cultures used in previous Northern blot experiments, in which a low level of *patB* message was observed at 3 to 6 h after nitrogen step-down, were pregrown in nitrate, which permits a low level of differentiation (14).

There are no recognized DNA regulatory elements in the immediate 5′ region of *patB*. The nearest clearly identifiable upstream ORF is *fdxB*, which is 990 bp upstream of *patB*. This ORF is oriented in the divergent direction and is expressed primarily in heterocysts (data not shown). *fdxB* has ≥58% similarity at the amino acid level to ferredoxin III proteins from many bacteria (1).

Expression of the heterocyst regulators *hetR* and *patS* is the same in the wild type and in *patB* mutants. Expression of the HetR master regulator of heterocyst development is both necessary and sufficient for this differentiation process (3, 4). However, the expression pattern of *hetR* can be regulated by other heterocyst-specific regulatory genes (such as *hetN*) that are themselves dependent upon *hetR* for expression (6). In the wild type, low-level GFP fluorescence from a *hetR*::*gfp* reporter is observed in whole filaments of nitrate-grown *Anabaena* sp. strain PCC 7120, although no fluorescence appears in cells in which heterocyst differentiation has been completely repressed by growth in 1 mM $(NH_4)_2SO_4$ (data not shown). After transfer to N_o medium, *hetR*::*gfp* expression is up-regulated in single proheterocysts (6). In contrast, in a strain underexpressing the *hetN* gene, *hetR* is up-regulated in multiple contiguous cells that have the appearance of proheterocysts, within 20 h of nitrogen removal (6). It was hypothesized that *hetR* expression might not resolve to single cells in a *patB* mutant and might serve as an indicator of partial heterocyst differentiation of the heterocyst-adjacent cells, prior to development of multiple contiguous heterocysts. However, during heterocyst differentiation in the *patBfr* mutant, there was no expression of a *hetR*::*gfp* transcriptional fusion in heterocyst-adjacent cells in the first few days after transfer to N_o medium. Therefore, a defect in *patB* has no effect on the single proheterocyst-specific induction of *hetR* expression in nitrogen-deprived cultures.

The *patS* gene encodes a heterocyst differentiation-inhibitory peptide (22). After removal of combined nitrogen from wild-type cultures, *patS* expression initially occurs in clusters of cells which are subsequently resolved to single proheterocysts spaced approximately 10 cells apart (22, 23). Expression of the *patS*::*gfp* reporter was examined in the *patB*fr mutants. Since, in the wild type, *patS* expression is up-regulated in clusters of cells and then resolved to single proheterocysts, it was thought that the *patS*::*gfp* reporter might serve as a finer indicator than *hetR*::*gfp* of partial heterocyst differentiation of the heterocyst-adjacent cells in *patB* mutants. However, it was determined that in the *patB*fr mutant, and all other *patB* mutants that have been constructed, *patS*::*gfp* expression is normal in the first 2 to 3 days after transfer to N_o medium, with expression resolving to single proheterocysts (Fig. 2B). After 3 days, *patB*fr carrying the *patS*::*gfp* reporter shows strong fluorescence from one or the other cell that is adjacent to a heterocyst (Fig. 2D). Expression of the *patS*::*gfp* reporter in the cell adjacent to filament-terminal heterocysts is seen in some wild-type filaments, but in *patB*fr, this heterocyst-adjacent-cell expression occurs

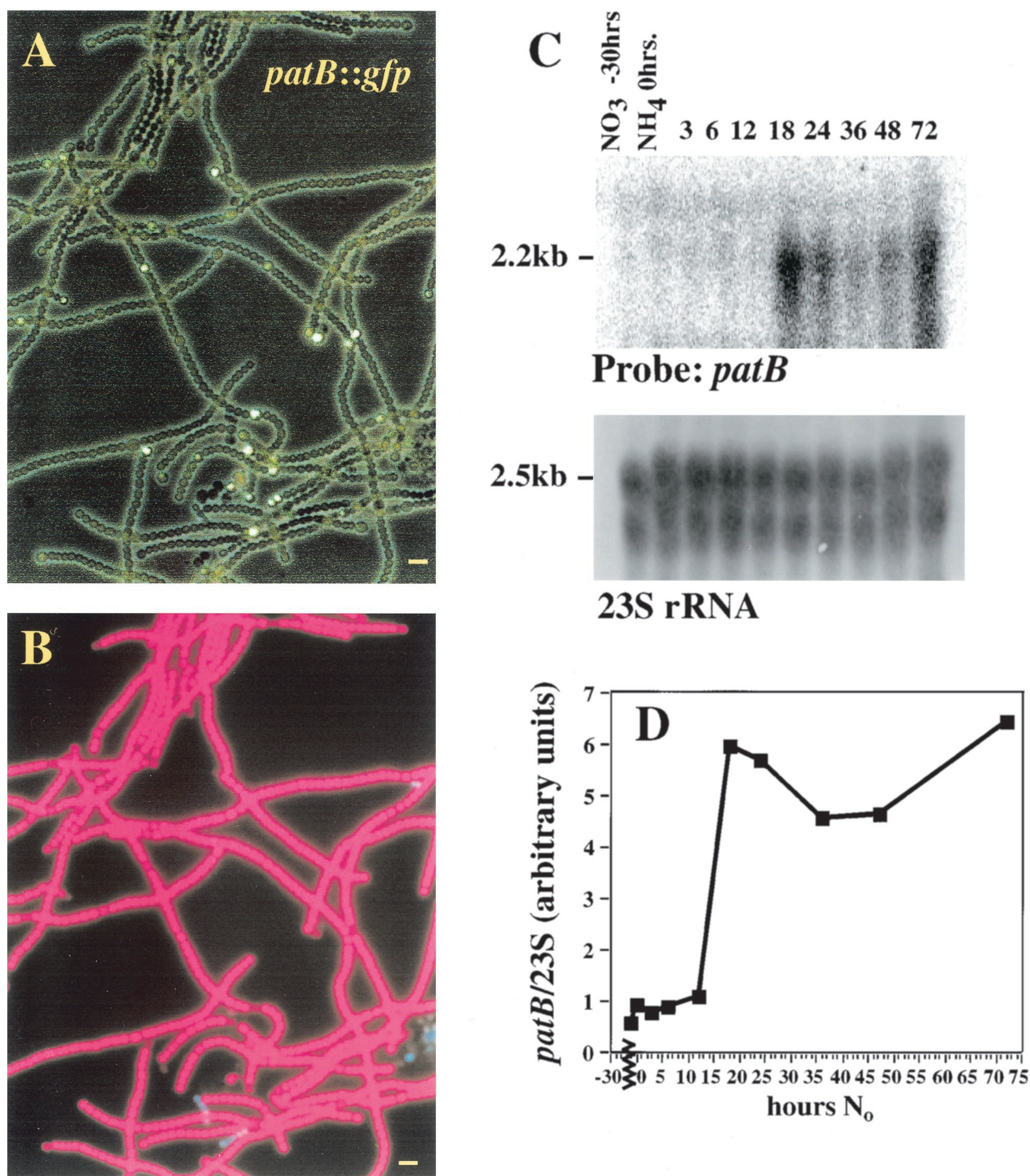

FIG. 1. (A) Composite image of *Anabaena* sp. strain PCC 7120 filaments carrying the *patB*::*gfp* transcriptional fusion at 51 h after nitrogen step-down, photographed under phase contrast, overlaid with the same field photographed with 450- to 490-nm excitation and emission through a 510-nm narrow-band-pass filter. Fluorescence is confined to the heterocysts. (B) Emission from chlorophyll visualized under 450- to 490-nm excitation, viewed without the emission filter. Heterocysts are the dim cells along the filament. The bars correspond to a length of 10 μm. (C) The upper panel shows a Northern blot of RNA prepared from wild-type *Anabaena* sp. strain PCC 7120 cells, following nitrogen step-down, probed with a *patB* internal fragment. The lower panel shows the bipartite 23S rRNA bands from a scan of the blots prior to hybridization, as described in Materials and Methods. These bands were quantified and used to normalize the phosphor signal for specific mRNAs from that lane (8). (D) The graph shows the *patB* signal from the time course (filled squares) normalized to 23S rRNA. The 2.2-kb message is induced 3.5-fold during the 12- to 18-h interval after removal of combined nitrogen.

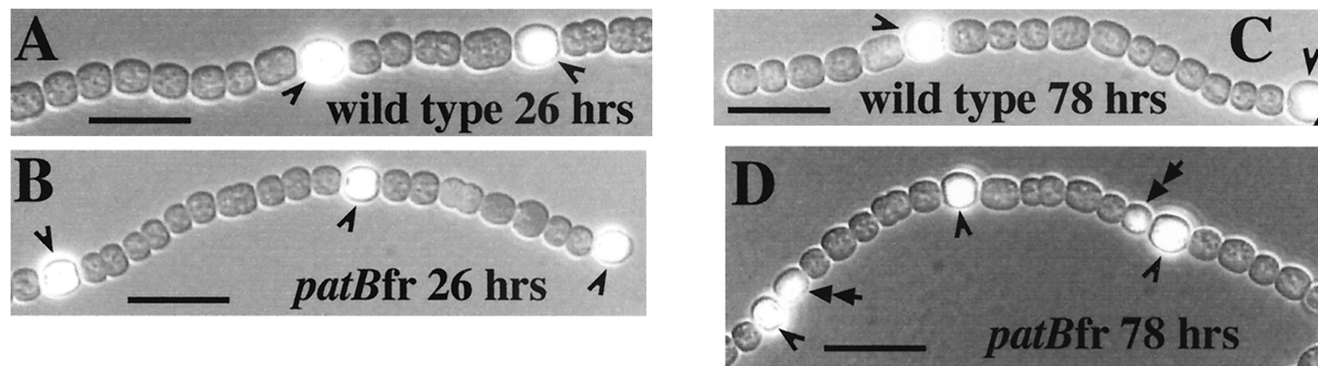

FIG. 2. Composite images of wild-type *Anabaena* sp. strain PCC 7120 filaments (A and C) and *patB*fr mutants (B and D) carrying the *patS*::*gfp* reporter (22) photographed under phase contrast, combined with the same microscopic field photographed with excitation and emission wavelengths of 450 to 490 nm and 510 nm, respectively. After 26 h in N_o medium, in wild-type (A) or *patB*fr (B) filaments, *patS*::*gfp* is expressed exclusively in heterocysts or proheterocysts (single arrowheads). After 78 h in N_o medium, the reporter is expressed exclusively in heterocysts of the wild type (C), but in *patB*fr (D), expression also occurs in heterocyst-adjacent cells (double arrowheads).

frequently at intercalary heterocysts as well. This is consistent with a model in which the gene expression cascade of early heterocyst development is not altered in *patB* mutants, but a failure of heterocyst function (see below) leads to further heterocyst differentiation.

Ectopic expression of *patB* from a heterologous promoter. The heterocyst differentiation regulators *patS* and *hetN* inhibit heterocyst differentiation when overexpressed and result in an increased frequency of heterocysts when underexpressed (6, 22). Since a null mutant of *patB* forms multiple contiguous heterocysts, it was hypothesized that overexpression of *patB* might also negatively regulate heterocyst development. However, a heterocyst suppression phenotype was not observed for filaments overexpressing *patB* (data not shown) from the copper-regulated promoter *petE* carried on a plasmid (p*petE*::*patB* plasmid) (data not shown) (10). Some cells in filaments ectopically expressing *patB*, in the absence of combined nitrogen, acquire some characteristics of heterocysts in an apparently random pattern. These cells form large polar granules and in some cases lose pigment fluorescence, events that occur during heterocyst development. However, these abnormal cells do not form the thickened double-layer cell walls characteristic of heterocysts.

Phenotypes of *patB* mutants. New mutants of *Anabaena* sp. strain PCC 7120 with defects in the *patB* gene were constructed, and their heterocyst pattern phenotypes (Fig. 3) and N_o growth phenotypes (Fig. 4) were analyzed. Three mutants were compared in this study: the previously characterized *patB* C-terminal frameshift mutant (*patB*fr), a deletion mutant of *patB* (*patB*Δ), and a mutant in which the putative iron-sulfur center cysteines were changed to alanines (*patB*FeS).

The *patB*fr mutant was previously determined to accumulate multiple contiguous heterocysts and to have delayed heterocyst formation, requiring 48 h rather than the normal 21 to 24 h for mature heterocysts to appear (14). The timing of heterocyst accumulation in the *patB*fr mutant was analyzed to determine whether multiple contiguous heterocysts develop immediately after the initial round of heterocyst formation or at a later time. The data presented in Table 1 demonstrate that the *patB*fr mutant does not accumulate a striking number of multiple contiguous heterocysts until 4 to 6 days after transfer to N_o medium. After 8 days, the *patB*fr mutant has >4-fold the number of multiple contiguous heterocysts as the wild type. Heterocysts that occur in clusters appear to be at different stages of development (Fig. 3). Older heterocysts of the *patB*fr mutant, as defined by apparently complete heterocyst walls and a large, rounded morphology, have a more light-colored, translucent appearance than normal heterocysts and are usually devoid of polar granules (Fig. 3B) (14). Filaments of this strain also fragment in the absence of combined nitrogen, as do those of many other mutants that are unable to grow in N_o (5, 14). Therefore, the initial round of heterocyst differentiation in the *patB*fr mutant has a normal pattern. The delay in the appearance of mature heterocysts in the *patB*fr mutant is the only abnormality in initial pattern formation.

The frameshift mutation in the *patB*fr mutant results in the loss of only the C-terminal portion of the protein. This C-terminal segment includes a domain with similarity to the HTH-3/HTH-XRE family of phage DNA-binding proteins. It was hypothesized that if PatB functions primarily as a DNA-binding protein with regulatory activity, the *patB*fr strain might be essentially a null mutant. To test this possibility, the deletion mutant (*patB*Δ), in which the entire ORF has been replaced by a Sm^r/Sp^r Ω cassette, was constructed. The defects of the *patB*Δ mutant differ from those of the *patB*fr mutant in several respects. The *patB*Δ mutant does not experience the delay in heterocyst differentiation of the *patB*fr strain. The *patB*Δ mutant resembles the *patB*fr mutant in that it has a normal initial heterocyst pattern, but it accumulates multiple contiguous heterocysts more rapidly than the *patB*fr mutant. After only 48 h in N_o medium, the *patB*Δ mutant begins to form what appear to be heterocyst clusters (Fig. 3C). After 7 days, the filaments in these cultures are so fragmented that very few filaments longer than 10 cells remain, and multiple contiguous heterocyst frequencies cannot be counted (Fig. 3D and Table 1). The defects of *patB*Δ can be complemented by expression of *patB* from the copper-regulated promoter *petE* carried on a plasmid (p*petE*::*patB* plasmid) (10).

Comparison of the severe effect that deletion of *patB* has on N_o-grown cultures with the milder phenotype of the C-terminal *patB* frameshift suggested that an N-terminal or central domain of the protein might be involved in an essential func-

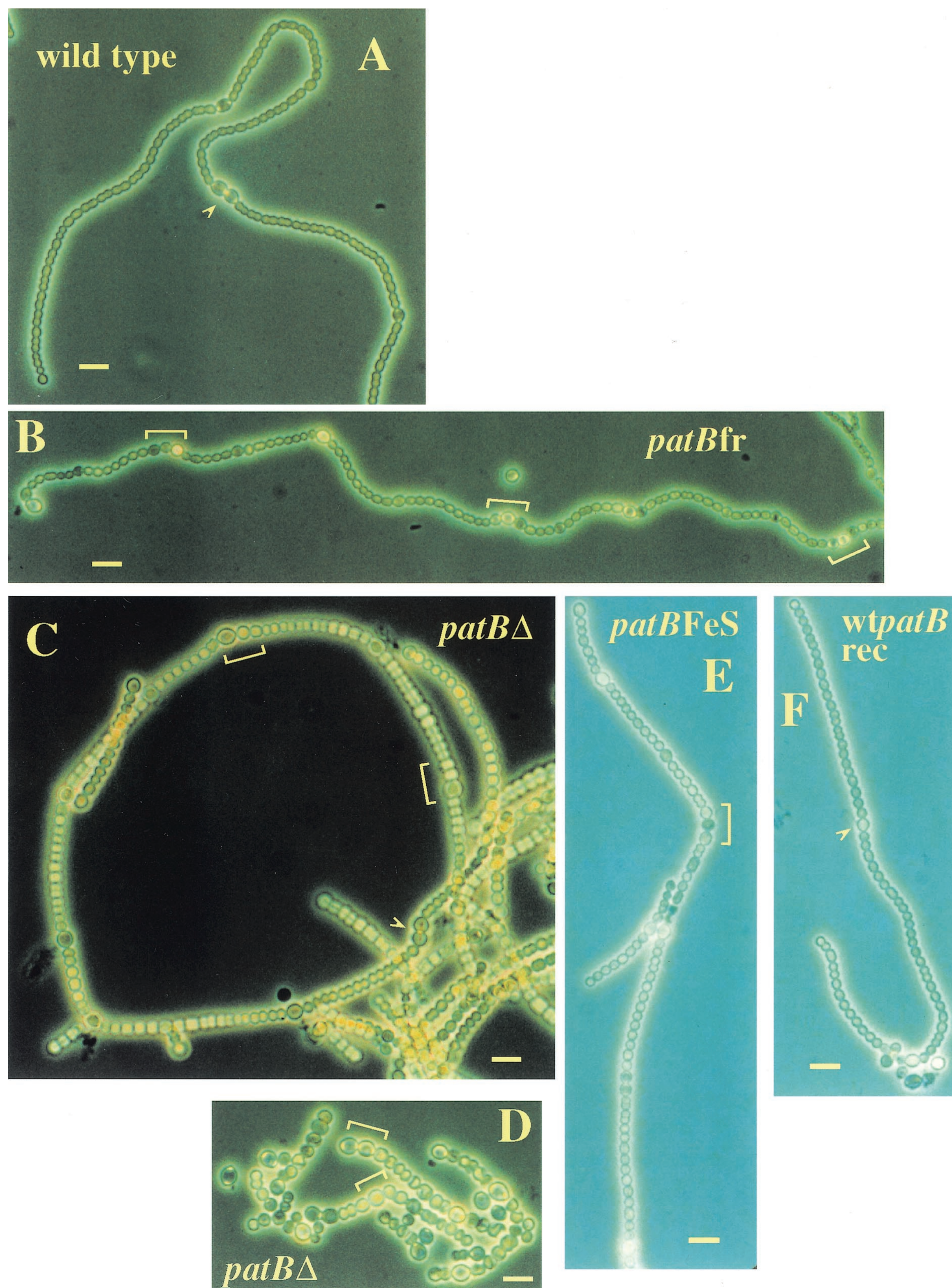

FIG. 3. Phase contrast images of the wild type (A) and the *patB*fr mutant (B) after one week of growth in N_o medium. The wild type has a double heterocyst (arrowhead) that looks very different from the groups of heterocysts accumulated in the *patB*fr filaments (brackets). After 48 h in N_o medium, the *patB*Δ mutant (C) is already accumulating groups of immature heterocysts (brackets). After 1 week in N_o medium, the *patB*Δ mutant (D) forms multiple contiguous heterocysts, but due to the extent of fragmentation, they are mostly at the ends of filaments. The *patB*FeS mutant (E) is compared with its wild-type sibling wt*patB*rec strain (F). Fewer clusters of multiple contiguous heterocysts (brackets) form in the *patB*FeS strain than in the other *patB* mutants. Bar, 10 μm.

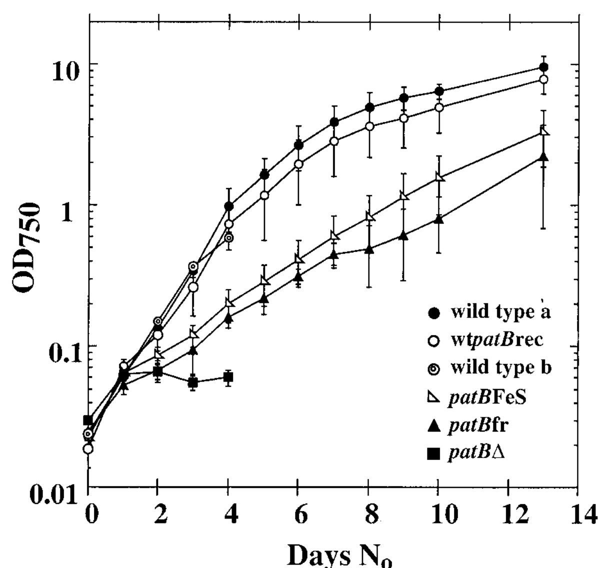

FIG. 4. Growth of the *patB* mutants in the absence of combined nitrogen, measured by absorbance at OD_{750}. The *patB*Δ mutant stops growing within 24 h of nitrogen removal. (The *patB*Δ results are an average of those for two replicates each of two separate isolates.) The growth of the *patB*fr mutant is less impaired than that of the *patB*Δ mutant, with growth continuing at a low rate for more than 2 weeks. (The *patB*fr results are averages of those for two replicates.) The growth of the *patB*FeS mutant is even closer to that of the wild type. This is consistent with the observed appearance of the filaments of this strain (see Fig. 3). (*patB*FeS results are averages for three replicates each of three separate isolates.) The wild-type wt*patB*rec reconstituted strain that is the sibling of the *patB*FeS strain grows almost as well as wild-type *Anabaena* sp. strain PCC 7120. wild type a, average of results for two replicates grown at the same time as the *patB*fr and *patB*FeS cultures. wild type b, average of results for two replicates grown at the same time as the *patB*Δ cultures.

tion. The N-terminal 58-amino-acid segment of PatB contains two putative 4Fe-4S centers and is 53% similar to several bacterial-type ferredoxin II proteins (1, 14). To test the possibility that the mutant phenotypes of *patB*Δ strains are caused primarily by the loss of these Fe-S centers, site-directed mutant strains were constructed in which the four cysteines of the first putative Fe-S center and the two 3′ cysteines of the second center were all changed to alanines. Constructs (pFeS*patB*replace or pwt*patB*replace) containing either the mutagenized *patB* ORF or the wild-type *patB* ORF, with 1 kb of wild-type flanking homology on either side, were conjugated into the *patB*Δ6 strain. Following initial selection with neomycin and sucrose counterselection against the *sacB* gene contained in the vector, the exconjugants were screened for loss of the Sm^r/Sp^r Ω cassette of the deletion mutant. *patB*FeS filaments do not exhibit the delayed heterocyst formation seen in *patB*fr filaments, and although they accumulate multiple contiguous heterocysts, the clusters are not as extensive as those of the *patB*fr mutant (Fig. 3E). The appearance of older heterocysts of the *patB*FeS and *patB*fr mutants is similar. The heterocysts are light-colored, translucent, and usually lack polar granules. The defects of *patB*FeS can be complemented by expression of *patB* from the p*petE*::*patB* plasmid (10). Also, the sibling control strain of the *patB*FeS mutant in which the Ω cassette of the deletion mutant was replaced with a wild-type *patB* ORF (wt*patB*rec) appears similar to wild-type *Anabaena* sp. strain PCC 7120 (Fig. 3F). These data indicate that the defects in the *patB*FeS mutant are due to the mutation of the cysteines to alanines. The ferredoxin-like domain of PatB is therefore required for full function of the protein, although the lesion in the *patB*FeS strain is much less severe than that of the deletion mutant and slightly less severe than that of the *patB*fr mutant.

The severity of the growth phenotypes of the *patB* mutants correlates with the degree of heterocyst clustering and filament fragmentation of these strains. The *patB*fr and *patB*FeS mutants are able to grow slowly in the absence of combined nitrogen for at least 2 weeks, while growth of the *patB*Δ mutant ceases after only one day (Fig. 4) (14). At the time that growth of *patB*Δ cultures stops, multiple contiguous heterocysts have not yet formed (Fig. 3C and 4).

A small amount of acetylene reduction activity (0.79 ± 0.34 pmol of acetylene reduced/h/optical density at 750 nm (OD_{750}) of culture) can be detected in the *patB*Δ mutant, indicating that a nitrogenase enzyme with at least some function is produced in this strain. This is a >390-fold reduction from the wild-type rate of 309 ± 53 pmol of acetylene reduced/h/OD_{750} of culture. Nitrogenase function in the *patB*fr mutant is reduced only sevenfold (41.7 ± 3.8 pmol of acetylene reduced/h/OD_{750} of culture).

Expression of a heterocyst-specific cytochrome *c* oxidase operon in *patB* mutants. An operon encoding a heterocyst-specific cytochrome *c* oxidase (*coxBACII*) is located 1.8 kb upstream of the *patB* start codon (11). The proximity of an operon encoding a putative heterocyst-specific terminal oxidase to *patB*, whose domain structure suggests that it might encode a redox-sensitive transcription factor, led us to investigate the mRNA levels of *coxBACII* in the *patB* mutants.

RNA was prepared from wild-type and *patB*fr cultures following transfer to N_o and hybridized with a gene-specific probe (a 0.16-kb *coxBII-coxAII* intergenic segment). The 3.8-kb message recognized by this probe increases 35-fold in the wild type,

TABLE 1. Timing of heterocyst accumulation

Genotype	% of heterocysts in clusters (*n*)[a]				
	1.5 days	3.5 days	5.5 days	6.5 days	8.5 days
Wild type	4.3 (15/352)	13.0 (85/645)	8.1 (43/533)	10.3 (57/555)	5.8 (50/858)
*patB*fr	7.2 (21/292)	14.2 (91/642)	20.5 (165/807)	22.2 (197/886)	25.3 (238/941)
*patB*Δ	7.2 (13/181)				

[a] *n*, mutiplex heterocysts/total heterocysts × 100. A multiplex heterocyst is defined as a contiguous cluster of heterocysts. For example, a filament with one single heterocyst and a contiguous cluster of three heterocysts would be counted as 1 multiplex/1 multiplex + 1 single. Only intercalary heterocysts were counted. Filament terminal heterocysts were excluded because heterocyst pattern is often aberrant at filament termini in all *Anabaena* sp. strain PCC 7120 strains.

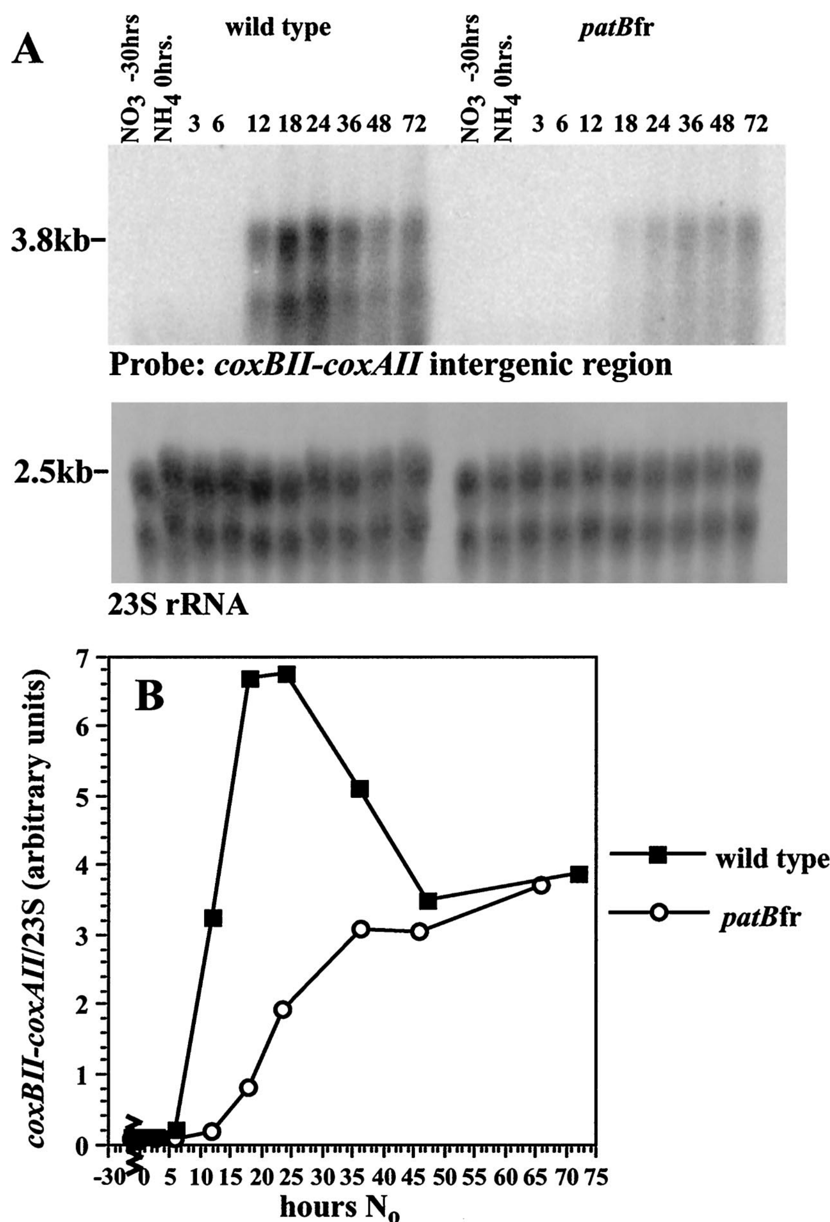

FIG. 5. Expression of the heterocyst-specific *coxBACII* operon in *patB* mutants. (A and B) RNA from nitrogen-starved cultures of the wild type and the *patB*fr mutant were probed with a 0.16-kb *coxBII-coxAII* intergenic fragment. The signal from this 3.8-kb message was normalized to the 2.5-kb 23S rRNA band. (C and D) RNA from two different *patB*Δ mutants was compared to that of the wild type, using the *coxBII-coxAII* probe and rRNA normalization described above. hours N_o, hours grown in medium free of combined nitrogen.

between 6 and 18 h after transfer (Fig. 5A and B). The *coxBACII* message level is depressed significantly in the *patB*fr mutant, especially at the earlier times. In contrast to these results obtained with the *patB*fr mutant, the *coxBII-coxAII* message levels in the deletion mutants of *patB* closely resemble those of the wild type. The results for *patB*Δ mutants 7 and 9 are shown in Fig. 5C and D. Unlike the *patB*fr mutant, the *patB*Δ mutants have an expression pattern of the 3.8-kb *coxBACII* message that is nearly identical to that of the wild type.

DISCUSSION

The *patB* frameshift mutant strain was originally identified on the basis of its very slow growth in the absence of fixed nitrogen (14). The sequence of the gene suggested that the protein had ferredoxin-like domains near the N terminus and a DNA-binding domain near the C terminus. These features suggested further that PatB might be a redox-sensitive transcription factor. In the present work, we show that *patB* expression is confined to heterocysts, that a mutant with a dele-

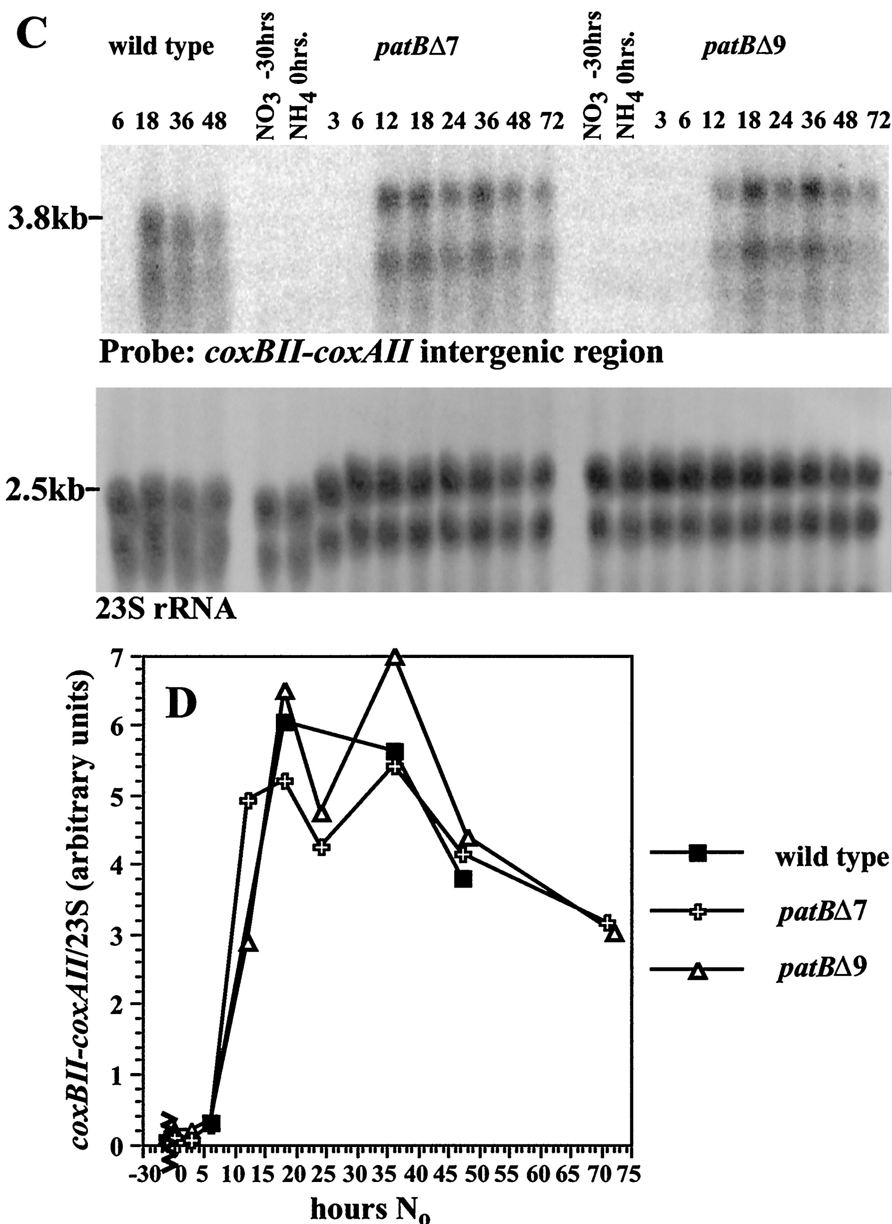

FIG. 5—*Continued*.

tion of *patB* has a more severe growth and nitrogen fixation defect than the original frameshift mutant, and that mutation of the putative ferredoxin domain also produces a growth and nitrogen fixation defect.

Expression of the master heterocyst regulator *hetR* is the same in *patB* mutants and in the wild type (data not shown), implying that unlike the *hetN* regulator, *patB* does not exert an effect on *hetR* expression. Also, expression of the heterocyst differentiation inhibitor *patS* is the same in the wild type and in *patB* mutants during the first 2 to 3 days after removal of combined nitrogen.

Although *patB* does not appear to be involved in the early-to-middle stages of the heterocyst differentiation process, it is required for survival in the absence of combined nitrogen. A deletion of the *patB* ORF results in cessation of growth in N_o medium within 24 h, only trace amounts of nitrogenase activity, rapid accumulation of multiple contiguous heterocysts, and fragmentation. These defects are more severe than those suffered in N_o medium by either a *patB* C-terminal frameshift mutant lacking the putative helix-turn-helix domain or an N-terminal mutant lacking the cysteine residues that would be required to form the putative Fe-S centers. Both of these mutants are capable of slow growth and slowly accumulate multiple contiguous heterocysts. The fact that these defects can be caused by site-directed mutations in six of the eight cysteines that would be critical for formation of an Fe-S center gives weight to the proposal that this is indeed an Fe-S domain.

The pairing of an Fe-S domain and a helix-turn-helix DNA

binding motif in a single ORF initially suggested that PatB might be a functional analog of FixK, a member of the fumarate and nitrate reductase (FNR) family of redox regulators that serves as an activator of genes required for symbiotic nitrogen fixation, including genes encoding a respiratory terminal oxidase (2, 16). However, PatB has significant differences from FNR family proteins in both its C-terminal helix-turn-helix domain and its N-terminal 4Fe-4S domain (1, 2, 12, 17). Also, the completion of the *Anabaena* sp. strain PCC 7120 genome has revealed that there are five ORFs in this organism (*Anabaena* sp. strain PCC 7120 genome database, Kazusa DNA Research Institute) with more extensive similarity to *E. coli* FNR and rhizobial FixK proteins than that possessed by PatB (1, 13).

If PatB is a transcriptional regulator binding DNA via the helix-turn-helix motif, the frameshift mutant would be expected to be a null. One possible explanation for the greater viability of *patB*fr than of *patB*Δ is that the frameshift mutation of *patB*fr that removes the putative DNA-binding domain might be leaky, allowing a low level of read-through, full-length protein. Even in the wild type, induction of the *patB* message in N_o cultures never attains a high level, requiring long exposure times of Northern blots to detect it. Also, the defects of the *patB* mutants can be complemented by the very low level of *patB* message that is produced from the copper-regulated *petE*::*patB* construct when no extra copper has been added to the growth medium (data not shown). A control construct containing the *petE* promoter without the *patB* ORF is not able to complement (data not shown). Therefore, the production of even a few copies of full-length protein might provide sufficient PatB function.

It is also possible that PatB has a function(s) that it is still able to perform in the absence of this C-terminal DNA-binding motif. If the N-terminal, putative Fe-S center is responsible for such a function, a *patB*fr/*patB*FeS double mutant would be expected to have an additive effect and result in a phenotype similar to that of the deletion mutant. Alternatively, another segment of the *patB* ORF might be required for its proper function. While there are no strongly conserved domains in the central 390 amino acids of the *patB* ORF, there are three short segments each of which has a low degree of similarity to protein sequences in GenBank (1). However, these similarities do not provide clues to PatB function (1). Further definition of the critical domains of PatB may be provided by a comparison of a *patB*fr/*patB*FeS double mutant with the *patB*Δ mutant and by the creation of mutations in the central portion of the *patB* ORF.

ACKNOWLEDGMENTS

This work was supported by research grant GM 21823 from the NIH and training grant GM O7183 from the NIH.

We thank James Golden and Ho Sung Yoon for DNA constructs and advice about GFP, Jeffrey Elhai for shuttle vectors, Jennifer Moran and Sean Callahan for helpful discussions, and Sheeba Thomas, Helene Callahan, Rama Malik, Anthony Gray, and Karalee Ensing for DNA sequencing.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Batut, J., M. L. Daveran-Mingot, M. David, J. Jacobs, A. M. Garnerone, and D. Kahn.** 1989. *fixK*, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. EMBO J. **8:**1279–1286.
3. **Buikema, W. J., and R. Haselkorn.** 1991. Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* 7120. Genes Dev. **5:**321–330.
4. **Buikema, W. J., and R. Haselkorn.** 2001. Expression of the *Anabaena hetR* gene from a copper-regulated promoter leads to heterocyst differentiation under repressing conditions. Proc. Natl. Acad. Sci. USA **98:**2729–2734.
5. **Buikema, W. J., and R. Haselkorn.** 1991. Isolation and complementation of nitrogen fixation mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **173:**1879–1885.
6. **Callahan, S. M., and W. J. Buikema.** 2001. The role of HetN in maintenance of the heterocyst pattern in *Anabaena* sp. PCC 7120. Mol. Microbiol. **40:** 941–950.
7. **Colon-Lopez, M. S., D. M. Sherman, and L. A. Sherman.** 1997. Transcriptional and translational regulation of nitrogenase in light-dark- and continuous-light-grown cultures of the unicellular cyanobacterium *Cyanothece* sp. strain ATCC 51142. J. Bacteriol. **179:**4319–4327.
8. **Eykholt, R. L., M. D. Mitchell, and K. W. Marvin.** 2000. Direct imaging of Northern blots on an optical scanner using ethidium bromide. BioTechniques **28:**864–866, 868, 870.
9. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of gram-negative bacteria. Gene **52:**147–154.
10. **Jones, K. M.** 2001. Ph.D. thesis. University of Chicago, Chicago, Ill.
11. **Jones, K. M., and R. Haselkorn.** 2002. Newly identified cytochrome *c* oxidase operon in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 specifically induced in heterocysts. J. Bacteriol. **184:**2491–2499.
12. **Jordan, S. R., and C. O. Pabo.** 1988. Structure of the lambda complex at 2.5 A resolution: details of the repressor-operator interactions. Science **242:**893–899.
13. **Kaneko, T., Y. Nakamura, C. P. Wolk, T. Kuritz, S. Sasamoto, A. Watanabe, M. Iriguchi, A. Ishikawa, K. Kawashima, T. Kimura, Y. Kishida, M. Kohara, M. Matsumoto, A. Matsuno, A. Muraki, N. Nakazaki, S. Shimpo, M. Sugimoto, M. Takazawa, M. Yamada, M. Yasuda, and S. Tabata.** 2001. Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. DNA Res. **8:**205–213.
14. **Liang, J., L. Scappino, and R. Haselkorn.** 1993. The *patB* gene product, required for growth of the cyanobacterium *Anabaena* sp. strain PCC 7120 under nitrogen-limiting conditions, contains ferredoxin and helix-turn-helix domains. J. Bacteriol. **175:**1697–1704.
15. **Murry, M. A., and C. P. Wolk.** 1989. Evidence that the barrier to the penetration of oxygen into heterocysts depends upon two layers of the cell envelope. Arch. Microbiol. **151:**469–474.
16. **Nellen-Anthamatten, D., P. Rossi, O. Preisig, I. Kullik, M. Babst, H. M. Fischer, and H. Hennecke.** 1998. *Bradyrhizobium japonicum* FixK2, a crucial distributor in the FixLJ-dependent regulatory cascade for control of genes inducible by low oxygen levels. J. Bacteriol. **180:**5251–5255.
17. **Padmanabhan, S., M. A. Jimenez, C. Gonzalez, J. M. Sanz, G. Gimenez-Gallego, and M. Rico.** 1997. Three-dimensional solution structure and stability of phage 434 Cro protein. Biochemistry **36:**6424–6436.
18. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Sigalat, C., and Y. Kouchkowski.** 1975. Fractionnement et caracterisation de l'algue bleue unicellulaire *Anacystis nidulans*. Physiol. Veg. **13:**243–258.
21. **Tandeau de Marsac, N., and J. Houmard.** 1988. Complementary chromatic adaptation: Physiological conditions and action spectra. Methods Enzymol. **167:**318–328.
22. **Yoon, H. S., and J. W. Golden.** 1998. Heterocyst pattern formation controlled by a diffusible peptide. Science **282:**935–938.
23. **Yoon, H. S., and J. W. Golden.** 2001. Pats and products of nitrogen fixation control heterocyst pattern. J. Bacteriol. **183:**2605–2613.