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Independent Regulation of *nifHDK* Operon Transcription and DNA Rearrangement during Heterocyst Differentiation in the Cyanobacterium *Anabaena* sp. Strain PCC 7120

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The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 expresses the genes required for nitrogen fixation in terminally differentiated cells called heterocysts. The *nifHDK* operon encodes the nitrogenase polypeptides and is expressed at high levels in heterocysts. During heterocyst differentiation, an 11-kb DNA element is excised from the *nifD* gene by site-specific recombination. The *xisA* gene, located on the 11-kb element, is required for the excision of the element. Transcription and DNA rearrangement of the *nifHDK* operon both occur late during heterocyst differentiation, about 18 to 24 h after induction, suggesting that the regulation of these events might be coupled. We show that heterocyst-specific transcription and DNA rearrangement of the *nifHDK* operon are independent of one another. Northern (RNA) analysis of the *xisA* mutant strain DW12-2.2, which cannot excise the *nifD* 11-kb element or fix nitrogen, showed that the *nifH* and *nifD* genes are transcribed on unrearranged chromosomes. The *nifK* gene was not transcribed in DW12-2.2, indicating that its expression is dependent on the *nifH* promoter and excision of the 11-kb element from the operon. A 1.68-kb DNA fragment containing the *nifH* promoter was deleted from the chromosome to produce the mutant strain LW1. LW1 formed heterocysts but did not grow on nitrogen-free medium and showed no transcription through *nifD*. Southern analysis of LW1 showed normal excision of the 11-kb element from the *nifHDK* operon, indicating that transcription from the *nifH* promoter is not required for the developmentally regulated DNA rearrangement.

The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 is capable of fixing atmospheric dinitrogen under aerobic conditions in terminally differentiated cells called heterocysts (22, 39). During conditions of combined-nitrogen deprivation approximately 1 in 10 photosynthetic vegetative cells will differentiate into a heterocyst. Heterocyst morphology and physiology are specialized for producing the anaerobic environment required for nitrogen fixation. Heterocyst differentiation is accompanied by substantial changes in gene expression (15, 30), including the expression of the *nifHDK* operon (23, 24). The *nifH* gene encodes dinitrogenase reductase, and *nifD* and *nifK* encode the alpha and beta subunits of dinitrogenase, respectively. Comparison of promoter sequences utilized in vegetative cells or heterocysts indicates that different forms of RNA polymerase may be active in the different cell types (6–8, 34, 38). Although heterocyst development has been well described (1, 22, 39) and heterocyst-specific genes that are expressed at different developmental times have been identified and cloned (6, 7, 11, 14, 26), little is currently known about the mechanism of temporal gene regulation during heterocyst differentiation. A cascade of sigma factors like that found during endospore formation in *Bacillus subtilis* (36) is a probable control mechanism, but conclusive evidence to support this hypothesis is still lacking. The development of a sensitive luciferase reporter system should stimulate research in this area (14, 40).

Two developmentally regulated DNA rearrangements occur near the nitrogen fixation genes during heterocyst differentiation of *Anabaena* sp. strain PCC 7120 (19, 23). One of these rearrangements involves the nitrogen fixation operon *nifHDK*. Relatively late during heterocyst differentiation, an 11-kb DNA element is excised as a circle from the coding region of the *nifD* gene by site-specific recombination between 11-bp directly repeated sequences present at the ends of the element (19). The excision results in formation of the complete *nifD* coding sequence and allows expression of the three genes, including the downstream *nifK* gene, as an operon transcribed from a promoter upstream of *nifH* (23). The gene *xisA*, located at one end of the *nifD* 11-kb element, is required for the excision of the element and is thought to encode a site-specific recombinase (9, 20, 28). Analysis of *xisA* transcription has been hampered because of the inability to detect mRNA transcripts, but DNA-protein interactions in the *xisA* upstream region probably play a role in its developmental regulation (9, 13). The *xisA* gene or its product must be tightly regulated because its activity, seen as the excision of the *nifD* element, is normally expressed only during the late stages of heterocyst differentiation (19).

The other DNA rearrangement is the excision of a 55-kb element from within the *fdxN* gene, which is part of the *nifB-fdxN-nifS-nifU* operon (17–19, 33, 34). Like the *nifD* rearrangement, the *fdxN* rearrangement is the result of site-specific recombination between terminal direct repeats (18), but the two rearrangements can occur independently under some inducing conditions (18) and the *xisA* gene is not involved in the *fdxN* rearrangement (20). Little is known about the mechanism of developmental regulation for either the DNA rearrangements or the transcription of the *nif* operons in which the elements reside.

We suspected that the developmental regulation of the transcription and DNA rearrangement of the *nifHDK* operon might be linked so that one of the events was dependent on the other. It seemed likely to us that the elements may derive their developmental regulation from the operon in which

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they reside, so that transcription of the operon triggers excision of the element. This would allow removal of the element when expression of the surrounding sequences was required by the host organism. We tested this hypothesis by separately blocking each of the events, transcription or rearrangement, by an appropriate genetic manipulation and then determining if the other event occurred normally. Our results showed that although the heterocyst-specific transcription and DNA rearrangement of the *nifHDK* operon have very similar developmental timing, each event occurred independently of the other.

MATERIALS AND METHODS

Strains and culture conditions. *Anabaena* sp. strain PCC 7120 was originally obtained from Robert Haselkorn. The *xisA* mutant strain DW12-2.2 was constructed in previous work (20). The *nifH* deletion strain LW1 is from this work. *Anabaena* strains were grown in 100-ml BG-11 (3) liquid cultures with shaking at 30°C with 60 to 80 $\mu E\ m^{-2}\ s^{-1}$ of illumination or on plates of BG-11 solidified with 1.5% agar at 30°C with 120 to 150 $\mu E\ m^{-2}\ s^{-1}$ of illumination. Photosynthetically active irradiance was measured with a Biospherical Instruments QSL-100 Laboratory Quantum Scalar Irradiance Meter. Larger-scale cultures (1 to 8 liters) were grown in the liquid medium of Allen and Arnon (2), diluted eightfold (AA/8), with the following modifications: the K_2HPO_4 concentration was doubled to 0.5 mM; the nickel, chromium, tungsten, and titanium salts were omitted; for AA/8 containing combined nitrogen, the medium was supplemented with MOPS [3-(*N*-morpholino)propanesulfonic acid] (5 mM, pH 8.0) and ammonium nitrate (2.5 mM). The larger-scale cultures were grown at approximately 30°C with illumination at approximately 100 $\mu E\ m^{-2}\ s^{-1}$ and bubbled with 1% CO_2 in air. Heterocyst formation was asynchronously induced by growth in liquid or solid BG-11 medium lacking sodium nitrate or synchronously induced in 1- to 8-liter cultures by transfer of mid-exponential-growth-phase cultures to AA/8 liquid medium lacking ammonium nitrate and MOPS.

Escherichia coli strains were maintained in LB liquid or on LB agar medium (Lennox L). For plasmid preparation, strains were grown in 0.5× TB liquid medium, a variation of Terrific Broth (37) containing (per liter) 100 ml of KH_2PO_4 (0.17 M)–K_2HPO_4 (0.72 M), 6 g of peptone, 12 g of yeast extract, and 2 ml of glycerol. Media were supplemented with appropriate antibiotics at standard concentrations (5). Plasmids were maintained in *E. coli* DH5α-MCR (Bethesda Research Laboratories, Life Technologies, Inc.).

LW1 construction. The *nifH* deletion strain LW1 was obtained by double recombination between the *Anabaena* sp. strain PCC 7120 chromosome and the nonreplicative plasmid pAM581. pAM581 (see Fig. 4) was constructed by first cloning the 8-kb *Eco*RI-*Sca*I fragment from pAn154 (34, 35), which contains sequences upstream of the *nifH* promoter, into *Eco*RI-*Eco*RV-digested pDW6 (20) to yield pAM544. pDW6 is an Ap^r conjugal nonreplicative vector based on pBR322. Then, the 9.9-kb *Kpn*I fragment of cosmid S2A12 (17), which contains sequences downstream of the *nifH* gene, was blunted by treatment with the Klenow fragment of DNA polymerase I and cloned into the *Eco*RV site of pBluescript KS+ (Stratagene) to produce pAM573. The insert was removed from pAM573 with *Bam*HI and *Sal*I and cloned into the same sites of pAM544, resulting in pAM579. A *Bam*HI Sm^r Spc^r Ω fragment from pDW9 (20) was cloned into the *Bam*HI site of pAM579 to produce pAM581.

The conjugation of the nonreplicative plasmid pAM581 and the selection for double recombinants were done essentially as described previously (20). By chance, the exconjugant colony picked for subcloning from the initial conjugation of pAM581, which was expected to be a single recombinant, was in fact a double recombinant. Subclone 19 (see Fig. 5, lane 3) from this original colony was selected as LW1. Single-recombinant clones were obtained from a different exconjugant colony from the initial conjugation.

Heterocyst isolation. Heterocysts from wild-type or mutant strains were purified from induced filaments essentially as previously described (19) with minor modifications. Vegetative-cell filaments from a late-exponential-growth-phase 3-liter AA/8 culture were collected by centrifugation and transferred to 1.5 liters of AA/8 medium lacking nitrate. Filaments from a 200-ml sample of the original culture were collected by centrifugation and frozen for the isolation of vegetative-cell DNA and RNA. The induced culture was grown for approximately 48 h before the heterocyst-containing filaments were collected by centrifugation at 4°C. A portion (approximately 1/15) of the induced filaments were collected and frozen for the isolation of DNA and RNA from whole filaments. The remaining cell pellet (3 to 4 ml) was suspended in 15 ml of 4°C STET (8% sucrose, 5% Triton X-100, 50 mM EDTA [pH 8.0], 50 mM Tris-HCl [pH 8.0]) containing lysozyme (1 mg/ml) in a plastic 50-ml centrifuge tube and vortexed vigorously for 2 to 3 min at room temperature. The viscosity of the solution was reduced by mild sonication with a Branson Sonifier Cell Disruptor 350 equipped with a tapered microtip at setting 3 for approximately 2 min on ice. Most of the vegetative cells lysed during this procedure, while the heterocysts remained intact. Heterocysts were collected by centrifugation at 3,000 × *g* for 5 min at 4°C. The heterocysts were washed once or twice by suspension in SET (8% sucrose, 50 mM EDTA [pH 8.0], 50 mM Tris-HCl [pH 8.0]) and centrifugation at 2,000 × *g* for 5 min at 4°C. The purified heterocysts were either processed immediately for the isolation of nucleic acids or were frozen at −90°C.

Nucleic acid methods. Standard methods were used for *E. coli* plasmid DNA isolations (essentially as described in the Geneclean kit; Bio 101, Inc.) and transformations (5). Restriction endonucleases and other DNA-modifying enzymes were used according to the manufacturer's recommendations or standard methods (5).

RNA and DNA were isolated from *Anabaena* filaments and purified heterocysts as described previously (19, 21). *Anabaena* genomic DNA minipreps were based on the procedure for the miniprep of bacterial genomic DNA described by Ausubel et al. (5). Filaments collected from 15 ml of a liquid culture or from a patch of cells scraped from the surface of solid media were transferred to a 1.5-ml microcentrifugation tube. Pelleted cells were suspended in 0.5 ml of STET containing lysozyme (1 mg/ml), vortexed vigorously for 2 min, and incubated at room temperature for 10 min. One-tenth milliliter of lysis mix (proteinase K [0.5 mg/ml] and 5% Sarkosyl) was added, and the samples were incubated at 55°C for 15 to 30 min. One-tenth milliliter of 5 M NaCl was mixed into each sample, followed by addition of 80 μl of CTAB/NaCl solution (10% cetyltrimethylammonium bromide, 0.7 M NaCl) and incubation at 65°C for 10 min. The aqueous samples were extracted sequentially with an equal volume of chloroform-isoamyl alcohol (24:1), phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). The nucleic acids were precipitated with 0.6

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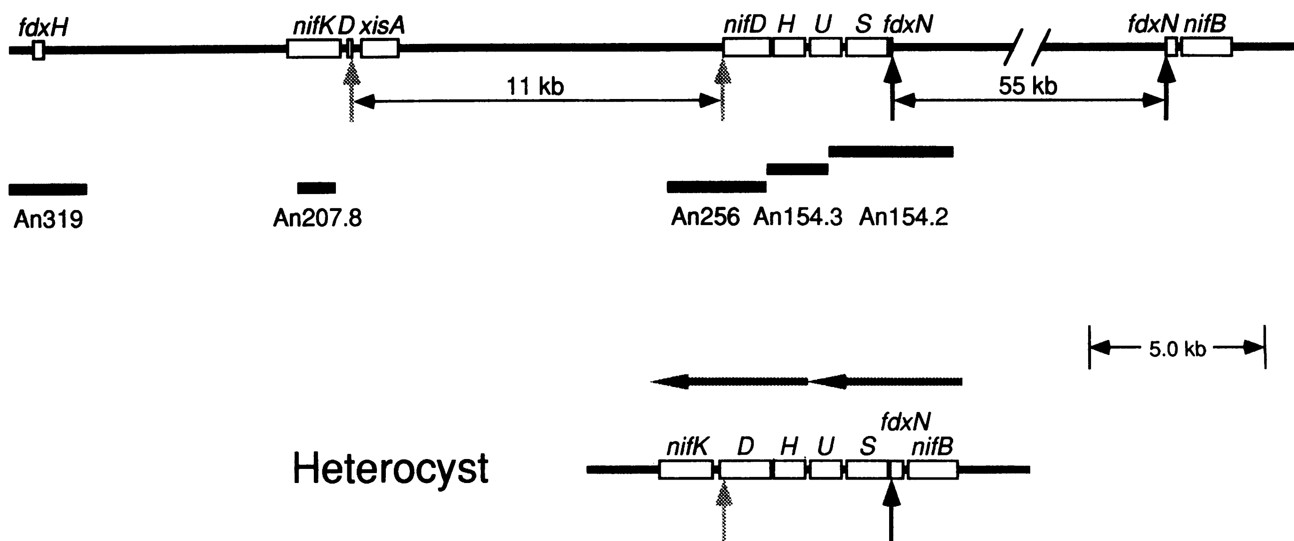

FIG. 1. Map of the *Anabaena* sp. strain PCC 7120 vegetative-cell and heterocyst chromosomal region containing nitrogen fixation genes and the *nifD* and *fdxN* DNA elements. The recombination sites that border the 11-kb *nifD* element and the 55-kb *fdxN* element are marked with grey and black vertical arrows, respectively. Open reading frames are shown as open rectangles and are labeled with their gene names. The *xisA* gene encodes the *nifD* element site-specific recombinase. In the heterocyst genome, two operons, shown as horizontal arrows, are formed by the excision of the DNA elements: a *nifHDK* operon and a *nifB-fdxN-nifS-nifU* operon. mRNA transcripts from the *nifHDK* operon promoter in unrearranged DNA would run into the right end of the *nifD* element. The excised DNA elements (not shown) are circularized during excision. Restriction fragments used as probes are labeled and shown as bars below the vegetative cell chromosome.

volume of isopropanol and collected by centrifugation. The nucleic acid pellets were dissolved in 200 μl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). One microliter of RNase (1 mg/ml) was added, and the samples were incubated for 30 min at 37°C. The DNA was precipitated with ethanol, washed with 70% ethanol, and dissolved in 20 μl of TE at 65°C for 10 to 15 min. This procedure typically yields several micrograms of genomic DNA.

DNA Southern transfers were from agarose gels run in TBE buffer (5); transfers were made to modified nylon membranes (GeneScreen Plus [Dupont, NEN Research Products] or MagnaGraph [MSI]) with 1 M NaCl–50 mM NaOH. Northern (RNA) transfers were from glyoxal or formaldehyde-agarose gels (5); transfers were made to modified nylon membranes with 10× SSPE (31). DNA probes were labeled by the random primer method, using a kit supplied by Boehringer Mannheim. Hybridization conditions were 5× SSPE–2% sodium dodecyl sulfate–sheared salmon sperm DNA (10 mg/ml) at 65°C, and membranes were washed in 0.5× SSPE–0.2% sodium dodecyl sulfate at 65°C.

RESULTS

Time course of *nifHDK* operon transcription. In *Anabaena* sp. strain PCC 7120 vegetative cells, the *nifHDK* operon is interrupted by an 11-kb DNA element inserted into the 3′ end of the *nifD* gene, and the *nifB-fdxN-nifS-nifU* operon is interrupted by a 55-kb DNA element inserted into the *fdxN* gene (Fig. 1). Both elements excise from the chromosome during the differentiation of a vegetative cell into a heterocyst. The *nifD* element excises from the chromosome by site-specific recombination between terminal 11-bp direct repeats. The product of the *xisA* gene is required for the rearrangement. In aerobically or anaerobically induced cultures, the *nifHDK* operon is transcribed in heterocysts (14, 17) from a single promoter (24) to produce three stable mRNA transcripts of 1.1, 2.8, and 4.7 kb containing the *nifH*; *nifH* and *nifD*; and *nifH*, *nifD*, and *nifK* open reading frames, respectively (23). Similarly, the *nifB-fdxN-nifS-nifU* operon is expressed only in induced cultures and produces at least three transcripts (34).

Rearrangements of the *nifHDK* operon and the *nifB-fdxN-nifS-nifU* operon are late events during heterocyst differentiation and are first detectable between 18 and 24 h after induction (19). During the same period proheterocysts can be identified morphologically and nitrogenase activity can be assayed. Transcription of the heterocyst-specific ferredoxin gene *fdxH* is first detectable at 18 h after induction (6). Elhai and Wolk showed that a *lacZ* gene driven by the *nifHDK* promoter in the heterocystless mutant *Anabaena* sp. strain PCC 7118 is first expressed between 13 and 16 h after anaerobic induction, with a significant increase in β-galactosidase activity after 18 h (14). Other genes have been shown to be expressed at earlier times during heterocyst differentiation: the *hetA* gene transcript shows its greatest abundance 7 h after induction (26), and *hetR* expression is significantly increased by 6 h after induction and peaks at 18 h (11).

Figure 2 shows the direct detection by Northern analysis of *nifHDK* transcripts at different times during aerobic induction of *Anabaena* sp. strain PCC 7120 in medium lacking a source of combined nitrogen. The An154.3 probe, which contains the *nifH* gene (Fig. 1), hybridized to each of the three stable *nifHDK* transcripts previously characterized (23). *nifHDK* mRNA was absent from vegetative cells,

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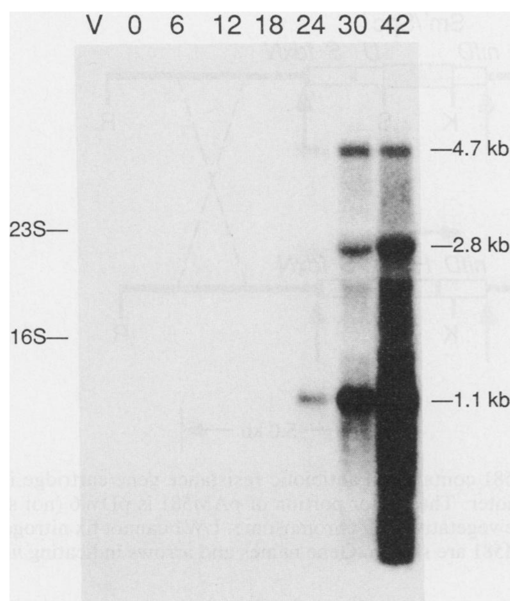

FIG. 2. Time course of *nifHDK* operon transcription during heterocyst differentiation. Total RNA from vegetative cells (V) and from induced filaments isolated 0, 6, 12, 18, 24, 30, and 42 h after transfer to nitrogen-free medium was run on a 1.2% agarose gel after treatment with glyoxal and was then blotted to a nitrocellulose membrane. The Northern blot was probed with the *Hin*dIII fragment An154.3, which contains the *nifH* gene and part of the *nifU* gene. The position of the 23S and 16S rRNA bands and the sizes of the three stable *nifHDK* operon transcripts are marked. The *nifB-fdxN-nifS-nifU* transcript is not detectable because of its low abundance.

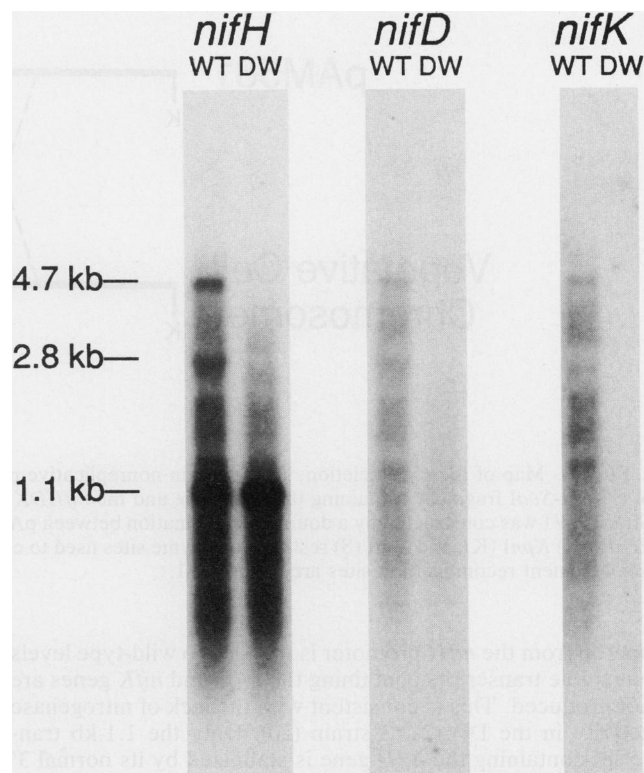

FIG. 3. Transcription of the *nifHDK* operon in DW12-2.2. Northern blots of RNA samples from wild-type (WT) and DW12-2.2 (DW) filaments induced on nitrogen-free medium and probed with labeled DNA fragments containing portions of the *nifH* (An154.3), *nifD* (An256), and *nifK* (An207.8) genes. Three stable transcripts are seen in the wild-type strain: a 1.1-kb transcript containing *nifH*, a 2.8-kb transcript containing *nifH* and *nifD*, and a 4.7-kb transcript containing *nifH*, *nifD*, and *nifK*. The DW12-2.2 strain shows only the stable 1.1-kb *nifH* transcript. A faint smear is seen with the *nifD* probe which probably represents unstable transcripts that run into the right end of the *nifD* element. The *nifK* probe does not detect any transcripts in DW12-2.2.

appeared between 18 and 24 h after induction, and showed the greatest abundance at the last sampling time of 42 h. Analysis of DNA isolated from the same samples showed excision of the *nifD* and *fdxN* elements between 18 and 24 h (19), the same time that transcription of the *nifHDK* operon begins. Therefore, the transcription and rearrangement of the *nifHDK* operon follow an identical developmental time course, indicating that the regulation of the two events may be directly coupled. We tested for regulatory dependence between the two processes by inactivating each process separately and then testing for the occurrence of the other process during heterocyst differentiation. Rearrangement of the *nifHDK* operon was blocked by inactivation of the *xisA* gene, and transcription of the operon was blocked by deletion of the operon's promoter.

Transcription of the unrearranged *nifHDK* operon. We have shown in previous work that inactivation of the *Anabaena xisA* gene prevents rearrangement of the *nifHDK* operon (20). The *xisA* gene was inactivated in strain DW12-2.2 by a double recombination event between the chromosome and a nonreplicative vector carrying a copy of the *xisA* gene interrupted by a Sm^r Spc^r Ω fragment. When induced on nitrogen-free medium, DW12-2.2 forms heterocysts but shows no nitrogenase activity and is unable to continue growth. The *nifD* element does not excise from the heterocyst chromosome in DW12-2.2, although the *fdxN* element excises normally (20).

Transcription of the *nifHDK* operon in an induced culture of DW12-2.2 was examined by Northern analysis (Fig. 3). Total induced RNA from the wild type and DW12-2.2 was hybridized with probes for each gene in the operon (Fig. 1): *nifH* (An154.3), *nifD* (An256), and *nifK* (An207.8). The wild type showed the expected stable transcripts of 1.1, 2.8, and 4.7 kb as well as a background smear of degraded transcripts. DW12-2.2 showed only the stable 1.1-kb transcript identified with the *nifH* probe, although this transcript was present at the normal abundance. The *nifH* probe also showed hybridization to a smear greater than 1.1 kb which apparently represents unstable transcripts that extend through *nifD* and into the 11-kb element. These transcripts would be antisense to the four open reading frames identified at this end of the *nifD* element (29). These longer transcripts would not contain the sequences that stabilize the normal 2.8- and 4.7-kb transcripts generated from the rearranged heterocyst chromosome. The *nifD* probe produced a very faint smear in DW12-2.2 induced RNA which presumably comprises the same unstable longer transcripts identified with the *nifH* probe. The *nifK* probe failed to hybridize to any transcripts in the DW12-2.2 induced RNA, indicating that the *nifH* promoter is unable to drive transcription of the *nifK* gene on the unrearranged chromosome and that there is no other promoter upstream of *nifK* that is utilized under our standard inducing conditions.

The results show that transcription of the unrearranged

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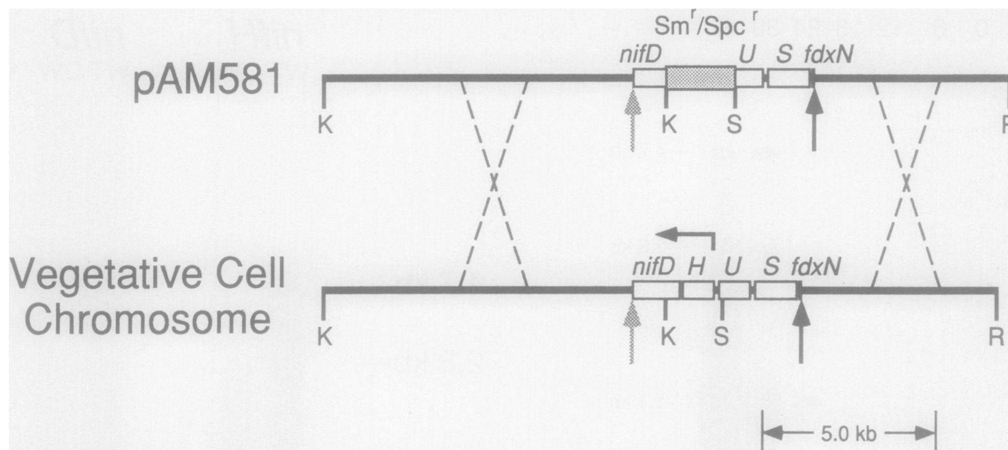

FIG. 4. Map of the *nifH* deletion. The insert in nonreplicative plasmid pAM581 contains an antibiotic resistance gene cartridge in place of a *KpnI-ScaI* fragment containing the *nifH* gene and the *nifHDK* operon promoter. The vector portion of pAM581 is pDW6 (not shown). Strain LW1 was constructed by a double recombination between pAM581 and the vegetative-cell chromosome. LW1 cannot fix nitrogen. The *EcoRI* (R) *KpnI* (K), and *ScaI* (S) restriction enzyme sites used to construct pAM581 are shown. Gene names and arrows indicating *nifD* and *fdxN* element recombination sites are as in Fig. 1.

operon from the *nifH* promoter is induced to wild-type levels but stable transcripts containing the *nifD* and *nifK* genes are not produced. This is consistent with the lack of nitrogenase activity in the DW12-2.2 strain (20). Only the 1.1-kb transcript containing the *nifH* gene is stabilized by its normal 3′ end, allowing for its accumulation.

Deletion of the *nifHDK* operon promoter. To determine if transcription from the *nifH* promoter is required to induce excision of the *nifD* 11-kb element, we created a 1.68-kb deletion in the chromosome which removed the *nifH* gene and the operon's promoter. The conjugal nonreplicative plasmid pAM581 was constructed so that a *KpnI-ScaI* fragment containing the *nifH* gene and its upstream sequences is replaced with a Sm^r Spc^r Ω fragment (Fig. 4). pAM581 contains large segments of flanking DNA, 10-kb and 8-kb fragments, to facilitate double recombination with the chromosome. The construction does not alter the recombination sites for either the *nifD* or *fdxN* element.

pAM581 was transferred into *Anabaena* sp. strain PCC 7120 by conjugation, and Sm^r Spc^r recombinants were selected. The initial recombinants were expected to be the result of a single crossover event between pAM581 and the chromosome and would be Ap^r (provided by the vector portion of pAM581) and capable of nitrogen fixation (assuming that the *nifD* and *fdxN* rearrangements could occur on the altered chromosome). Double recombinants would be Sm^r Spc^r but Ap^s and unable to fix nitrogen. Fragmented filaments from a culture started from an initial colony were plated with selection for Sm^r Spc^r, and the resulting secondary colonies were independently tested for Ap^r and their ability to grow in the absence of combined nitrogen. All tested colonies showed the phenotype expected for double recombinants, indicating that the initially selected colony was a double recombinant. A different colony from the initial conjugation that showed Ap^r and growth in the absence of combined nitrogen was selected as an example of a single recombinant.

Genomic vegetative-cell DNA was isolated from five putative double recombinants (numbered 1 to 5) and the single recombinant (no. 6) for Southern analysis (Fig. 5). *EcoRI*-digested DNA probed with An256 (Fig. 1) showed the expected 17-kb (An207) (35) and 0.82-kb (from the pAM581 insert) fragments for the double recombinants; the single recombinant showed the additional expected 10.5-kb (An154) (35) and 13-kb (from the pAM581 plasmid) fragments. *HindIII*-digested DNA probed with An256 showed a 2.6-kb fragment for the double recombinants, shortened from the normal 2.9 kb because of the Sm^r Spc^r Ω fragment in the pAM581 insert; the single recombinant showed both the normal 2.9-kb An256 fragment plus the altered 2.6-kb fragment. Genomic DNA from double and single recombinants digested with *EcoRI* or *HindIII* showed the expected 10.0- and 2.0-kb fragments, respectively, when probed with the Sm^r Spc^r Ω fragment. The 10.0-kb *EcoRI* fragment is from An154 and is shortened as a result of the incorporation of the Sm^r Spc^r Ω fragment. The 2.0-kb *HindIII* fragment is from the Sm^r Spc^r Ω fragment itself. The An154 *EcoRI* fragment was used to probe the same Southern blots shown in Fig. 5, and it also produced the expected fragments for the single and double recombinants (results not shown). The Southern analysis confirmed the deletion of the *nifH* gene and promoter in the double recombinants. The subcloned colony represented in Fig. 5, lanes 3, was selected for further analysis and was named strain LW1.

Northern analysis of induced RNA from strain LW1 was done to confirm that the deletion of the *nifH* promoter eliminated transcription through *nifD* into the 11-kb element (Fig. 6). Northern blots of total RNA from wild-type vegetative and induced filaments and LW1 induced filaments were hybridized with An256 (*nifD*) and An319 (*fdxH*) probes. The An256 probe detected the expected 4.7-kb (faint band) and 2.8-kb transcripts, as well as a smear of partially degraded transcripts, from the wild-type induced filaments but failed to detect any transcripts from the LW1 induced filaments. This result confirmed that the deletion had removed the *nifHDK* operon promoter. To show that the RNA isolated from LW1 induced filaments was not degraded and contained heterocyst-specific transcripts, a Northern blot was probed with An319 (shown in Fig. 1), which contains the *fdxH* gene (6). The An319 probe hybridized with the expected transcripts at 1.85 and 0.59 kb in both wild-type and LW1 induced RNA, confirming the quality of the RNA isolated from LW1. The increased abundance of the *fdxH* 1.85-kb transcript in the LW1 sample compared with that in

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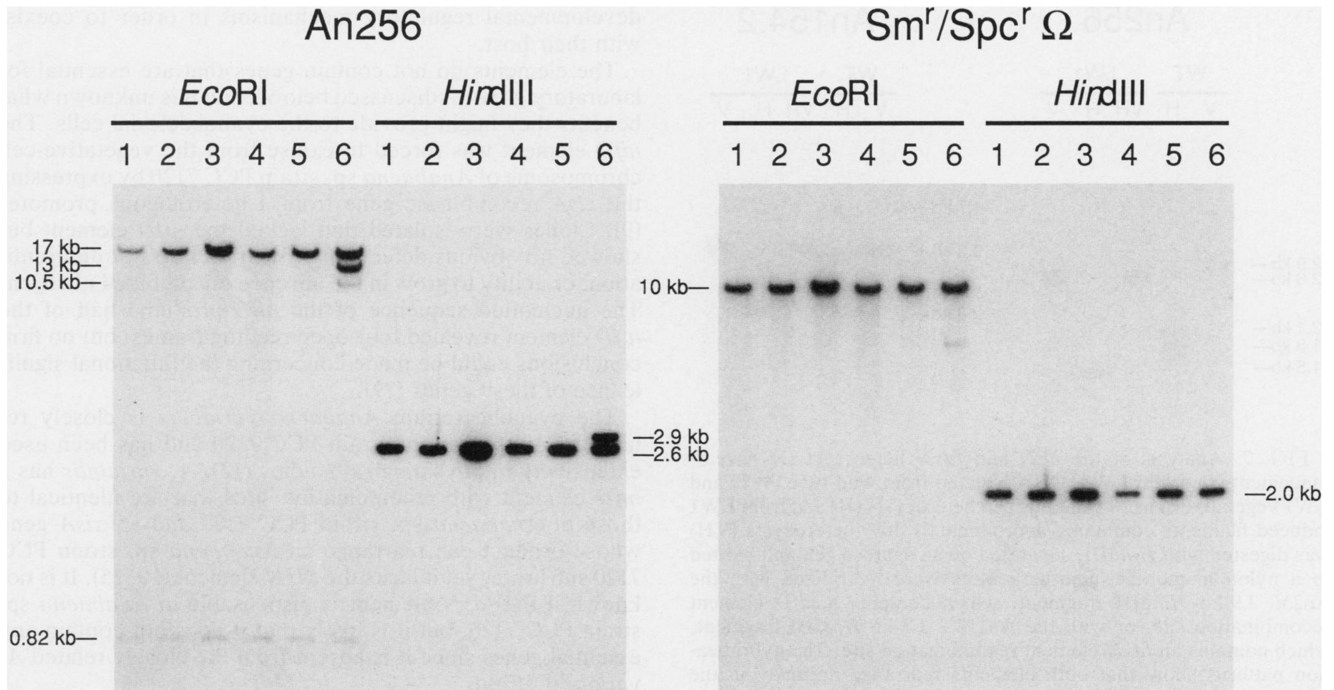

FIG. 5. Southern blot analysis of pAM581 single and double recombinants. DNA from double recombinants (lanes 1 through 5) and a single recombinant (lanes 6) was digested with *Eco*RI or *Hin*dIII, run on an agarose gel, and blotted to a nylon membrane. Southern blots were hybridized with a labeled An256 *Hin*dIII fragment, which contains *nifD*, or the Sm^r Spc^r Ω fragment from pAM165 (20). The hybridization patterns were as expected (see the text). Clone 3 was selected as the double recombinant strain LW1.

our wild-type sample and with the data obtained by others (6) is of unknown significance but is presumably related to the Nif$^-$ phenotype of LW1. No transcripts were detected in vegetative-cell RNA with either the *nifD* or *fdxH* probe.

Rearrangement of the promoterless *nifHDK* operon. The effect of the LW1 *nifHDK* promoter deletion on heterocyst-specific DNA rearrangements was examined by Southern analysis of *Hin*dIII-digested DNA isolated from vegetative cells and heterocysts and probed with DNA fragments which identify the *nifD* (An256) and *fdxN* (An154.2) rearrangements (Fig. 7). The An256 probe identified itself at 2.9 kb in wild-type vegetative-cell DNA and a 2.6-kb fragment in LW1, which is shortened by 0.3 kb because of the *nifH* deletion. Rearranged heterocyst DNA showed the normal 2.1- and 1.8-kb fragments for the wild type and the same 2.1-kb fragment and a shortened 1.5-kb fragment for LW1, showing that the *nifD* element had excised from the chromosome normally. The 2.1-kb band in the LW1 heterocyst sample was fainter than expected. This fragment represents the excised 11-kb element circle, and it is usually present in approximately equal amounts relative to the 1.8-kb chromosomal band (1.5-kb band in LW1). Although very faint in this exposure, equal levels of these two bands were present in the LW1 DNA isolated from induced total filaments (LW1 lane VH). The circular 11-kb DNA element may have been partially lost during the isolation of DNA from purified LW1 heterocysts.

The excision of the *fdxN* 55-kb element also was not affected by the deletion of the *nifHDK* promoter (Fig. 7). For both wild type and LW1, the An154.2 probe identified itself at 3.3 kb in vegetative cells and the rearranged 2.3- and 4.7-kb fragments in heterocyst DNA. Therefore, transcription of the *nifHDK* operon is not required for either of the two heterocyst-specific DNA rearrangements.

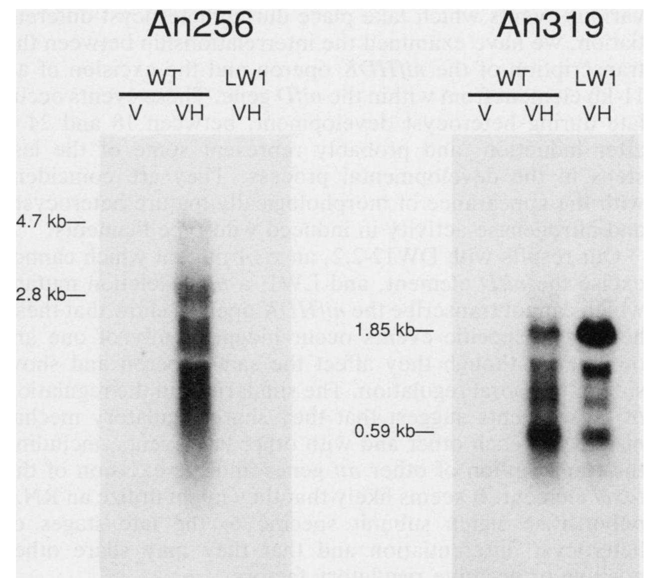

FIG. 6. Northern analysis of LW1. RNA from wild-type (WT) vegetative cells (V) and wild-type and LW1 induced filaments containing vegetative cells and heterocysts (VH) was separated on an agarose-formaldehyde gel and blotted to nylon membranes. The Northern blots were hybridized with the An256 *Hin*dIII fragment, which contains *nifD*, or with the An319 *Hin*dIII fragment, which contains the heterocyst-specific ferredoxin gene *fdxH*. Both probes hybridize to the expected transcripts in wild-type induced RNA and fail to identify transcripts in the vegetative-cell RNA. Although the LW1 RNA sample shows hybridization to the An319 control probe, it fails to hybridize with the *nifD* probe. Therefore, there is no transcription into the right end of the *nifD* element in the LW1 strain.

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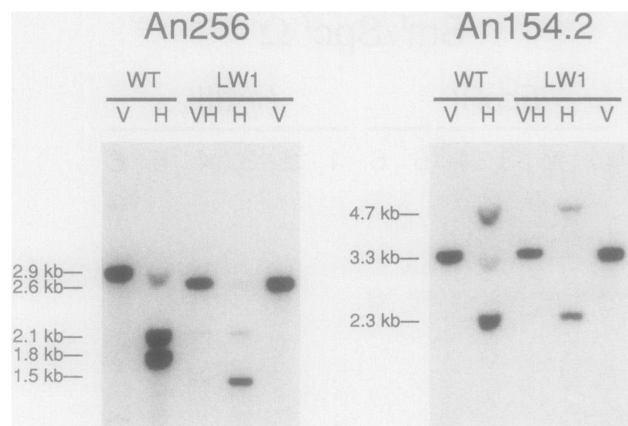

FIG. 7. Analysis of the *nifD* and *fdxN* heterocyst DNA rearrangements in strain LW1. DNA isolated from wild-type (WT) and LW1 vegetative cells (V) and purified heterocysts (H) and from LW1 induced filaments containing approximately 10% heterocysts (VH) was digested with *Hin*dIII, separated on an agarose gel, and blotted to a nylon membrane. Southern blots were hybridized with the An256 2.9-kb *Hin*dIII fragment, which contains a *nifD* element recombination site, or with the An154.2 3.3-kb *Hin*dIII fragment, which contains an *fdxN* element recombination site. The hybridization patterns show that both elements rearrange normally in the LW1 strain.

DISCUSSION

As part of our effort to understand the regulation of the various events which take place during heterocyst differentiation, we have examined the interrelationship between the transcription of the *nifHDK* operon and the excision of an 11-kb element from within the *nifD* gene. These events occur late during heterocyst development, between 18 and 24 h after induction, and probably represent some of the last steps in the developmental process. They are coincident with the appearance of morphologically mature heterocysts and nitrogenase activity in induced wild-type filaments.

Our results with DW12-2.2, an *xisA* mutant which cannot excise the *nifD* element, and LW1, a *nifH* deletion mutant which cannot transcribe the *nifHDK* operon, show that these heterocyst-specific events occur independently of one another even though they affect the same operon and show similar temporal regulation. The similarities in the regulation of these events suggest that they share regulatory mechanisms with each other and with other late events, including the transcription of other *nif* genes and the excision of the *fdxN* element. It seems likely that they might utilize an RNA polymerase sigma subunit specific to the late stages of heterocyst differentiation and that they may share other positive or negative regulatory factors.

It is clear why the excision of the two DNA elements is developmentally regulated; they must be correctly removed from the chromosome for proper expression of the *nif* operons within which they reside. This has been shown to be true for the *nifD* element in strain DW12-2.2 (20) and is probably true for the *fdxN* element. But it is not clear how the developmental regulation originated. Although we suspected that the excision of the elements might be regulated by the transcription of the operon in which they were inserted, the results presented here do not support that idea. If the elements are or once were temperate cyanophage, they must have evolved the ability to respond to the host's developmental regulatory mechanisms in order to coexist with their host.

The elements do not contain genes that are essential for laboratory growth (discussed below), and it is unknown what benefits they might provide to the cyanobacterial cells. The *nifD* element was forced to excise from the vegetative-cell chromosome of *Anabaena* sp. strain PCC 7120 by expressing the *xisA* recombinase gene from a heterologous promoter (9). Clones were isolated that lacked the *nifD* element but showed no obvious defects in growth, heterocyst differentiation, or ability to grow in the absence of combined nitrogen. The nucleotide sequence of the *nifH*-proximal half of the *nifD* element revealed four open reading frames, but no firm conclusions could be made concerning the functional significance of these genes (29).

The cyanobacterium *Anabaena variabilis* is closely related to *Anabaena* sp. strain PCC 7120 and has been used extensively in physiological studies (12). *A. variabilis* has a *nifD* element with recombination sites that are identical to those of *Anabaena* sp. strain PCC 7120 and an *xisA* gene whose product can rearrange an *Anabaena* sp. strain PCC 7120 substrate, yet it lacks the *fdxN* element (10, 25). It is not known if the *fdxN* element is dispensable in *Anabaena* sp. strain PCC 7120, but it is likely that it does not contain any essential genes since it is absent from the closely related *A. variabilis* strain.

The *nifD* element is fairly widespread among the heterocystous cyanobacteria (4, 10, 16, 19, 25, 27, 32). Lammers et al. have proposed that the elements have evolved to utilize sequences within the *nif* genes as integration sites because these sequences would be conserved among the nitrogen-fixing cyanobacteria (29). The *nif* integration sites would not affect any vegetative cell functions but require that the elements excise from the heterocyst chromosome. The mechanism of this developmental regulation remains to be determined.

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