The *sigE* Gene Is Required for Normal Expression of Heterocyst-Specific Genes in *Anabaena* sp. Strain PCC 7120$^{\nabla}$†

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The filamentous cyanobacterium *Anabaena* (*Nostoc*) sp. strain PCC 7120 produces specialized cells for nitrogen fixation called heterocysts. Previous work showed that the group 2 sigma factor *sigE* (alr4249; previously called *sigF*) is upregulated in differentiating heterocysts 16 h after nitrogen step-down. We now show that the *sigE* gene is required for normal heterocyst development and normal expression levels of several heterocyst-specific genes. Mobility shift assays showed that the transcription factor NtcA binds to sites in the upstream region of *sigE* and that this binding is enhanced by 2-oxoglutarate (2-OG). Deletions of the region containing the NtcA binding sites in P*$_{sigE}$*-*gfp* reporter plasmids showed that the sites contribute to normal developmental regulation but are not essential for upregulation in heterocysts. Northern RNA blot analysis of *nifH* mRNA revealed delayed and reduced transcript levels during heterocyst differentiation in a *sigE* mutant background. Quantitative reverse transcription-PCR (qRT-PCR) analyses of the *sigE* mutant showed lower levels of transcripts for *nifH*, *fdxH*, and *hglE2* but normal levels for *hupL*. We developed a P*$_{nifHD}$*-*gfp* reporter construct that showed strong heterocyst-specific expression. Time-lapse microscopy of the P*$_{nifHD}$*-*gfp* reporter in a *sigE* mutant background showed delayed development and undetectable green fluorescent protein (GFP) fluorescence. Overexpression of *sigE* caused accelerated heterocyst development, an increased heterocyst frequency, and premature expression of GFP fluorescence from the P*$_{nifHD}$*-*gfp* reporter.

Bacteria can respond to environmental and intracellular conditions by regulation of gene expression, often via changes in transcriptional initiation of specific genes. Transcriptional regulation in bacteria is associated with two major families of sigma factors: sigma 70 (σ^{70}), which is subdivided into four groups (19), and sigma 54 (σ^{54}), which has no clear homologs in cyanobacteria or in Gram-positive bacteria with high GC contents (48). Sigma factors are often key regulators in complex responses such as bacterial development and biofilm formation. For example, in *Bacillus subtilis*, temporally and spatially regulated sigma factors control the expression of genes during starvation-induced sporulation (7, 28), and in the Gram-negative bacterium *Pseudomonas aeruginosa*, sigma factors control the expression of antibiotic and phagocyte resistance genes during biofilm formation (42).

In cyanobacteria, sigma factors have been shown to be involved in a variety of regulatory responses (24, 38). In this paper, we present data showing that the *Anabaena* (*Nostoc*) sp. strain PCC 7120 (here *Anabaena* PCC 7120) *sigE* gene (alr4249; previously called *sigF*), which encodes a group 2 σ^{70}

family sigma factor, is required for the normal expression of some genes involved in heterocyst development. (We follow the revised nomenclature of Yoshimura et al. for cyanobacterial sigma factor names [3, 58].) The expression of *sigE* in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 increases after nitrogen depletion, and this response is dependent on the nitrogen regulator NtcA (35, 39); mobility shift assays using purified NtcA show an interaction with the *sigE* promoter region *in vitro*. Expression studies comparing a *sigE* null mutant with wild-type *Synechocystis* sp. strain PCC 6803 showed that the expression of one of the nitrogen assimilation genes, *glnN*, was impaired in the mutant strain (35). DNA microarray experiments with *Synechocystis* sp. strain PCC 6803 showed that the expression of genes involved in glycolysis, the oxidative pentose phosphate pathway, and glycogen catabolism was decreased in a *sigE* mutant strain, suggesting that *sigE* is involved in both nitrogen and sugar metabolism (41). Recent studies with *Synechocystis* sp. strain PCC 6803 showed that *sigE* is posttranslationally activated by the light-to-dark transition and that ChlH, the H subunit of Mg-chelatase, interacts with SigE *in vivo* and functions as an anti-sigma factor, transducing light signals to SigE in a process mediated by Mg^{2+} (40).

The multicellular cyanobacterium *Anabaena* PCC 7120 is a well-studied heterocyst-forming strain with tractable genetics and a fully sequenced genome. Heterocyst development is an established model for the study of bacterial differentiation, pattern formation, and nitrogen fixation (2, 17, 29). *Anabaena* PCC 7120 grows as long chains of photosynthetic vegetative cells in media containing combined nitrogen. In media lacking combined nitrogen, heterocysts

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differentiate from vegetative cells to form a semiregular pattern of single heterocysts every 10 to 20 vegetative cells along filaments. Heterocysts are capable of fixing nitrogen, which they supply to vegetative cells in the form of amino acids, and the vegetative cells supply fixed carbon to the heterocysts (17, 29). Heterocyst development can be synchronously induced by nitrogen step-down, i.e., the transfer of filaments to a medium lacking combined nitrogen.

Heterocysts are morphologically and physiologically differentiated from vegetative cells and exhibit large-scale changes in gene expression. One of the earliest steps in heterocyst differentiation is the increased expression of *ntcA*, which is an autoregulatory transcription factor belonging to the cyclic AMP (cAMP) receptor protein (CRP) family (22). NtcA together with HetR, a master regulator for heterocyst differentiation, directly or indirectly regulates the expression of many genes involved in heterocyst differentiation and the assimilation of atmospheric nitrogen (17, 29, 30, 36, 37, 54, 59). NtcA activity is modulated by 2-oxoglutarate (2-OG), which is an intermediate in the incomplete Krebs pathway present in cyanobacteria, and its increase constitutes the signal for nitrogen limitation.

During heterocyst differentiation, a polysaccharide layer is deposited around the proheterocyst, followed by the deposition of a glycolipid layer between the outer membrane and the polysaccharide layer (4). Synthesis and deposition of the polysaccharide layer requires the expression of a cluster of genes that constitute the HEP island (23). The expression of these genes is upregulated during the early stages of heterocyst differentiation. Synthesis of the glycolipid layer requires the expression of *hglB*, *hglC*, *hglD*, *hglE* (*hglE$_A$*), *hglK*, and *devBCA*, which are all upregulated early during heterocyst differentiation (4, 15, 25). A second cluster of *hgl* genes includes the *hglE2* gene (all1646), which shows *devH*-dependent upregulation after heterocyst induction (45). The morphologically differentiated heterocysts become micro-oxic partly because these layers limit oxygen diffusion into the heterocyst (16). In addition, heterocysts increase respiration to decrease oxygen levels (51).

Complete maturation of heterocysts involves the expression of genes in the late stages of heterocyst development that are necessary for nitrogen fixation and assimilation into amino acids. The *nifHDK* operon encodes the nitrogenase polypeptides and is upregulated 12 to 18 h after nitrogen step-down in heterocysts (14, 18). Other late heterocyst-specific genes include *fdxH*, which encodes a [2Fe-2S] ferredoxin (33), and *hupL*, which encodes the large subunit of uptake hydrogenase (9, 10). These genes are regulated during heterocyst development and potentially by oxygen or metabolite levels in heterocysts, but the factors required for the regulation of these late genes are not yet known.

The role of sigma factors in transcriptional regulation during heterocyst development has been studied for many years. Northern RNA blot analysis showed that two sigma factor genes, *sigB* and *sigC*, are upregulated 12 h after nitrogen step-down (6). However, single and double mutations of *sigB* and *sigC* showed that they are not essential for heterocyst differentiation or diazotrophic growth. In later work, insertional inactivation of *sigC*, *sigF*, *sigB2*, *sigD*, or *sigE* showed that the mutants form heterocysts and can grow on nitrate or diazo-

trophically but that *sigB2* and *sigD* mutants are significantly slow to establish diazotrophic growth (27). A *sigB2 sigD* double mutant can form proheterocysts but is unable to grow diazotrophically, possibly due to extensive fragmentation of filaments upon nitrogen deprivation (27). The failure to find any single sigma factor that is essential for heterocyst development suggests that the cyanobacterial group 2 sigma factors have partially overlapping functions, which has hampered genetic analysis (3, 27, 32, 39, 58).

A more recent study of *Anabaena* PCC 7120 used *gfp* transcriptional reporter fusions to analyze the spatial and temporal patterns of expression for all sigma factor genes located on the chromosome except *sigA* (3). These studies found that three sigma factor genes (two group 2 sigma factor genes, *sigC* and *sigE*, and one representative of group 4, *sigG*) are upregulated in heterocysts after induction of heterocyst development (3). Expression of *sigC* is upregulated by 4 h after nitrogen step-down, suggesting that it may be involved in the early stages of heterocyst development. The *sigG* gene is expressed in vegetative cells grown in nitrogen-containing media, but after nitrogen step-down its expression in vegetative cells decreases, and by 10 h individual differentiating cells show increased expression. These results suggest that SigG may be involved in the expression of middle-stage heterocyst genes in addition to genes in vegetative cells grown on nitrate. The expression of *sigE* is localized to differentiating heterocysts at around 16 h after nitrogen step-down, suggesting that SigE might be important for the regulation of genes that are expressed in the late stages of heterocyst development.

In this work, we present evidence that *sigE* is required for normal heterocyst development and the normal expression of the heterocyst-specific *nifH*, *fdxH*, and *hglE2* genes.

MATERIALS AND METHODS

Strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. *Anabaena* PCC 7120 and its derivatives were grown in BG-11 or BG-11$_0$, which lacks sodium nitrate, medium at 30°C with illumination of approximately 75 μmol photons m^{-2} s^{-1} from fluorescent lights as previously described (18, 46). *Escherichia coli* strains were grown in LB (Lennox L) medium containing appropriate antibiotics at 37°C. *E. coli* strain DH10B was used for all cloning experiments. Shuttle plasmids and suicide plasmids were transferred into *E. coli* donor strain AM1359 (56) by electroporation and transferred to *Anabaena* PCC 7120 strains by conjugation using standard protocols (13) with some modifications (27).

The *sigE* mutant strain AMC649 was revived from a frozen stock and confirmed to show the previously reported phenotype (27). In addition, the *sigE* expression plasmid pAM3961 was transferred by conjugation into AMC649, and the resulting strain showed complementation of the delayed heterocyst differentiation phenotype.

DNA manipulations and plasmid constructions. Standard protocols were used for cloning, *E. coli* transformation, and PCR. Primers used in this study are listed in Table 2. Total DNA from *Anabaena* PCC 7120 was isolated as previously described (27). DNA sequencing of plasmid inserts was performed by the Gene Technologies Laboratory (Texas A&M University) following the Big Dye sequencing protocol (Applied Biosystems) and by Genewiz (La Jolla, CA).

A *sigE* overexpression plasmid was constructed by PCR amplification of *sigE* (alr4249) with primers containing an NdeI site at the 5′ end and an XmaI site at the 3′ end (AMO-2222 and AMO-2223) and then ligation of the fragment into the same sites in pAM2770, which contains a copper-inducible *petE* promoter.

Site-directed mutagenesis of the consensus NtcA binding site in the *sigE* promoter was conducted using standard protocols (26). Primers AMO-2217 and AMO-2218 were used to mutate the site in pAM3652 (P$_{sigE}$-gfp).

Plasmids containing *gfpmut2* transcriptional fusions with *sigE* upstream fragments (pAM3652, pAM4466, pAM4467, pAM4468, and pAM4469) were constructed in shuttle plasmid pAM1956 (Table 1). The *sigE* upstream fragments P1,

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Anabaena strains		
PCC 7120	Wild-type *Anabaena* sp. strain PCC 7120	R. Haselkorn
AMC649	PCC 7120 *sigE*::*luxAB*-Sm^r/Sp^r cassette at ClaI site of *sigE*; Sm^r Sp^r	27
AMC1452	PCC 7120 carrying P$_{sigE}$-*gfpmut2* on pAM3652; Km^r Nm^r	3
AMC1773	PCC 7120 carrying P$_{petE}$-*sigE* on pAM3961; Km^r Nm^r	This study
AMC1774	PCC 7120 with integrated pRL277-P$_{nifHD}$-*gfpmut2*; Sm^r Sp^r	This study
AMC1775	PCC 7120 with integrated pRL277-P$_{nifHD}$-*gfpmut2* and P$_{petE}$-*sigE* on pAM3961; Sm^r Sp^r Km^r Nm^r	This study
AMC1776	PCC 7120 with integrated pRL278-P$_{nifHD}$-*gfpmut2*; Km^r Nm^r	This study
AMC1777	AMC649 carrying pRL278-P$_{nifHD}$-*gfpmut2*; Km^r Nm^r	This study
AMC1789	PCC 7120 carrying P$_{sigE-P1}$-*gfpmut2* on pAM4469; Km^r Nm^r	This study
AMC1790	PCC 7120 carrying P$_{sigE-P2}$-*gfpmut2* on pAM4468; Km^r Nm^r	This study
AMC1791	PCC 7120 carrying P$_{sigE-P3}$-*gfpmut2* on pAM4467; Km^r Nm^r	This study
AMC1792	PCC 7120 carrying P$_{sigE-P4}$-*gfpmut2* on pAM4466; Km^r Nm^r	This study
Plasmids		
pAM1956	Shuttle vector pAM505 containing promoterless *gfpmut2* with unique upstream cloning sites SalI, SacI, KpnI, ACC651, and SmaI; Km^r Nm^r	56
pAM2770	Shuttle vector containing XhoI-P$_{petE}$-NdeI-*lacZα*-SapI(Cys)-6His (stop)-ClaI; Km^r Nm^r	31
pAM3652	pAM1956 containing 800-bp fragment upstream of *sigE* (alr4249), P$_{sigE}$-*gfpmut2*	3
pAM3929	*ntcA* cloned into expression plasmid pET-28b+ in EcoRI and XhoI sites	This study
pAM3961	*sigE* (ORF alr4249) cloned into pAM2770 containing P$_{petE}$ in NdeI and XmaI sites; Km^r Nm^r	This study
pAM4466	pAM1956 containing 79-bp fragment upstream of *sigE* (alr4249), P$_{sigE-P4}$-*gfpmut2*; Km^r Nm^r	This study
pAM4467	pAM1956 containing 260-bp fragment upstream of *sigE* (alr4249), P$_{sigE-P3}$-*gfpmut2*; Km^r Nm^r	This study
pAM4468	pAM1956 containing 360-bp fragment upstream of *sigE* (alr4249), P$_{sigE-P2}$-*gfpmut2*; Km^r Nm^r	This study
pAM4469	pAM1956 containing 500-bp fragment upstream of *sigE* (alr4249), P$_{sigE-P1}$-*gfpmut2*; Km^r Nm^r	This study
pRL277	Conjugal suicide plasmid; Sm^r Sp^r	13
pRL278	Conjugal suicide plasmid; Km^r Nm^r	13

P2, P3, and P4 were PCR amplified from total genomic DNA with primers shown in Table 2. The resulting fragments contained engineered SalI and Acc65I sites and were inserted at the same sites in pAM1956.

Electrophoretic mobility shift assay (EMSA). A $6\times$ His-tagged NtcA protein was overexpressed from the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible plasmid pET-28b+ and purified using a HisTrap HP 1-ml nickel column (GE Healthcare) by standard procedures. A 207-bp DNA fragment upstream from the *sigE* gene containing a putative consensus NtcA binding site was amplified by PCR using oligonucleotides RAM-Fw and RAM-Rv, each containing an engineered EcoRI site at the 5′ end. The PCR product was digested with EcoRI and labeled with [α-^{32}P]dATP (3,000 Ci mmol^{-1}) by fill-in using the DNA polymerase Klenow fragment. Binding assays were carried out in a final volume of 10 μl containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 5 ng μl^{-1} bovine serum albumin, 12.5% (vol/vol) glycerol, and 10 fmol of the DNA fragment; the reaction mixture was supplemented with 2-oxoglutarate (0.6 mM) where indicated. The labeled probe was incubated with 1 to 4 pmol of purified $6\times$ His-NtcA protein for 30 min at room temperature. DNA fragments were separated by electrophoresis on a nondenaturing 5% polyacrylamide gel, vacuum dried, and visualized by phosphorimaging (Fujifilm). Negative controls for binding included DNA fragments from the *hetR* and *sigC* upstream regions (not shown).

RNA isolation. A RiboPure-Bacteria kit (Ambion) was used to isolate total RNA from *Anabaena* PCC 7120 and its derivates. One flask was grown for each time point sample for each strain. Filaments from growing BG-11 cultures were inoculated into 250-ml flasks containing 100 ml of BG-11(NH$_4^+$), which is BG-11$_0$ supplemented with 2.5 mM ammonium chloride and 5 mM MOPS (morpholineethanesulfonic acid) (pH 8.0) to obtain a starting optical density at 750 nm (OD$_{750}$) of 0.025 to 0.035. These cultures were grown on an orbital shaker under standard conditions overnight to an OD$_{750}$ of 0.05 to 0.075, and then the cells were collected at room temperature by centrifugation in 50-ml conical tubes, washed twice with BG-11$_0$, and transferred to 100 ml of BG-11$_0$ in 250-ml flasks. The flasks were incubated under standard conditions for the appropriate time after nitrogen step-down. Each culture was then poured into a conical 250-ml centrifuge tube containing 100 g of crushed ice to rapidly chill the

TABLE 2. DNA primers used in this study

Gene and expt	Forward primer		Reverse primer	
	Name	Sequence (5′→3′)	Name	Sequence (5′→3′)
sigE, probe	AMO-2222	CCTATTCCCGGGCTCAAATCAACCAACC	AMO-2223	AACAGTCATATGGAAATTATGTACCAAAC
sigE, upstream for EMSA	RAM-Fw	CTTGAATTCTAAAGATAAATATTCTTATTGCC	RAM-Rv	GTGGAATTCAAATATTTATTGCACGTAT
ntcA, overexpression	AMO-2026	CCGGAATTCATCGTGACACAAGATAAG	AMO-2027	TAACCTCGAGTTAAGTGAACTGTCTGCTG
P$_{sigE-P1}$	AMO-2558	GTCGTCGACTTTTATACAAACAATTTTGTG	AMO-2554	GGTGGTACCTTGAGGGATTCATGCTTT
P$_{sigE-P2}$	AMO-2557	GTCGTCGACTTTATAAAAGTATATAATTATT	AMO-2554	GGTGGTACCTTGAGGGATTCATGCTTT
P$_{sigE-P3}$	AMO-2556	GTCGTCGACAGATTAAATGATTAATAAA	AMO-2554	GGTGGTACCTTGAGGGATTCATGCTTT
P$_{sigE-P4}$	AMO-2555	GTCGTCGACATGAATCTGAAAATTGCT	AMO-2554	GGTGGTACCTTGAGGGATTCATGCTTT
nifH, probe	AMO-622	TTCACGTCAACCTTACGG	AMO-1038	CGGTAAAGGCGG
Site-directed mutagenesis of P$_{sigE}$-*gfp*	AMO-2217	GTATTTTGAATGCAGATTTTATTC	AMO-2218	GAATAAAATCTGCATTCAAAATAC
nifH, qRT-PCR	AMO-2185	GCACAAGAAATCTACATC	AMO-2186	TACGAAGTGAATCATTTG
hupL, qRT-PCR	AMO-2189	TGCTTCTCACTTAACCTCTG	AMO-2190	GTCAATGGCGAACAATCC
fdxH, qRT-PCR	AMO-2191	TACCAAGTTAGATTGATC	AMO-2192	AAGTAACACAAAGTAGAG
hglE2, qRT-PCR	AMO-2197	TTGTTGAGGTGTATTGAG	AMO-2198	CAGGAACATCAGTGATAC
rpoA, qRT-PCR	AMO-2204	AGTTTGGACAAATGGTAG	AMO-2205	GCTTGAGACAGTTATAGG

sample, and the filaments were collected by centrifugation at $5,000 \times g$ for 10 min at 4°C. The cell pellet was immediately frozen at -80°C and stored until RNA was isolated.

Northern RNA blot analysis. For each sample, 10 μg of total RNA was separated on a 1.5% agarose denaturing formaldehyde gel in MOPS buffer (47) and then transferred by capillary action to a Magnacharge nylon membrane (GE Osmonics) with 10× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH$_2$PO$_4$, and 1 mM EDTA [pH 7.7]) (18). DNA probes were amplified by PCR with appropriate primers (Table 2), labeled with [α-^{32}P]dCTP by random-primer labeling, and purified on Micro Bio-Spin P-30 columns (Bio-Rad). Blots were blocked, hybridized with radioactively labeled DNA probes, and washed using standard protocols (47) and then were exposed to a phosphorimager plate and scanned with a BAS-5000 phosphorimager (Fujifilm).

Time-lapse microscopy. Time-lapse microscopy was used to record differential interference contrast (DIC), autofluorescence of photosynthetic pigments, and green fluorescent protein (GFP) fluorescence images during heterocyst development. Filaments of each reporter strain were grown in nitrate-containing BG-11 medium to an OD$_{750}$ of 0.2 to 0.3, washed with BG-11$_0$, and resuspended in a small volume of BG-11$_0$ for heterocyst induction. Ten microliters of induced *Anabaena* filaments were placed into a single-chamber cover glass (Lab-Tek chamber slide system; Nalge Nunc International) and covered with a thin agarose pad supported by a dialysis membrane. The agarose pad was made by slicing open a dialysis tube (1.5 cm by 2.5 cm) (VWR Scientific; molecular mass cutoff, 3,500 kDa) that had been equilibrated in BG-11$_0$, laying the dialysis membrane on a glass slide, and then covering the membrane with 300 μl of warm filtered BG-11$_0$ containing 0.7% agarose. After the agarose cooled and solidified, small square sections (about 0.4 by 0.4 cm) of the agarose pad were cut and used to cover the induced filaments in the single-chamber cover glass. Excess liquid medium was removed, and the agarose pad was then carefully surrounded on all four sides by a total of about 2 ml of warm BG-11$_0$ containing 0.7% agarose in order to maintain moisture in the thin agarose pad. For time-lapse microscopy, the chamber was covered with its plastic lid.

Microscopy was performed with a DeltaVision Core system (Applied Precision) with a WeatherStation attached to an Olympus IX71 inverted microscope. The temperature was adjusted to 30°C. softWoRx software was used to acquire time-lapse images for 24 to 42 h with a 15-min time delay using a 40× objective. The time-lapse series was started approximately 10 to 20 min after nitrogen step-down. The *Anabaena* filaments were illuminated to support their growth during time-lapse microscopy with approximately 75 μmol photons m^{-2} s^{-1} from a fiber optic LED white light source (Schott IFC 60825) attached to the DeltaVision Core system and controlled by softWoRx software, which controlled switching between the external white light and the microscope white light and excitation light for taking images. For all images, a polychroic beam splitter was used. The DIC exposure time was 0.05 s. For autofluorescence images, a tetramethyl rhodamine isocyanate (TRITC) filter set was used (EX555/EM617) with an exposure time of 0.05 s, and for GFP images, a Chroma GFP filter set was used (HQ EX470/EM515) with a 0.5-s exposure time. Time-lapse images were processed using softWoRx software, and individual images from specific time points were exported as required.

Scoring of the heterocyst spacing pattern along filaments was performed essentially as previously described (57). Detached single heterocysts and aggregates of heterocysts were not scored. Because the strains AMC1774 and AMC1775 contained a *nifHD-gfp* reporter, GFP fluorescence was used as an additional character to identify proheterocysts. Strain AMC1777 was scored only by morphology because the *nifHD-gfp* reporter did not produce detectable GFP fluorescence in the *sigE* mutant background.

qRT-PCR analysis. Quantitative real-time reverse transcription-PCR (qRT-PCR) analysis was carried out with a StepOnePlus 96-well qRT-PCR system (Applied Biosystems) and Power SYBR green PCR master mix (Applied Biosystems) mixed with 100 pmol/μl of each primer and 50 ng of total cDNA. All primers used in this study are listed in Table 2. Primers for *nifH*, *fdxH*, *hupL*, *hglE2*, and *rpoA* were designed with Vector NTI software (Invitrogen), and each primer was analyzed using the BioBike web interface for sequence similarity searches against the *Anabaena* PCC 7120 genome. cDNA synthesis from total RNA samples (grown on nitrate or at 12, 24, and 36 h after nitrogen step-down) was performed with an iScript cDNA kit (Bio-Rad). All samples were run in triplicate using the following amplification conditions: 1 cycle of 2 min at 52°C and 10 min at 95°C and then 40 cycles of 95°C for 15 s and 60°C for 30 s. Cycle threshold (C_T) values for each gene were averaged and normalized against *rpoA*.
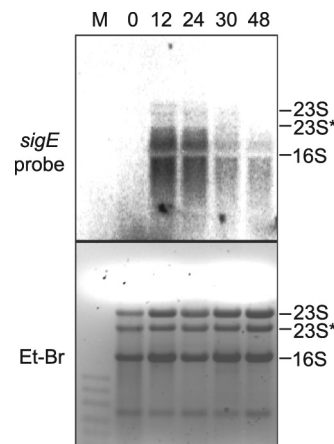

FIG. 1. Northern RNA blot analysis of *sigE* transcripts during heterocyst development. Lanes are labeled with hours after nitrogen step-down from BG-11 to BG-11$_0$ medium. The upper panel shows a Northern RNA blot probed with a radioactive probe for *sigE* transcripts. The lower panel shows the ethidium bromide (Et-Br)-stained gel to show sample loading. Lane M, low-range ssRNA ladder (NEB) size marker.

RESULTS

***sigE* transcripts.** Northern RNA blot analysis of *sigE* was performed to determine transcript size and abundance at different developmental times. The Northern RNA blot results showed that *sigE* was upregulated after nitrogen step-down and that transcript levels were highest at 12 and 24 h (Fig. 1). The *sigE* transcripts were present as a smear, suggesting a short half-life, with an upper edge at around 2.5 to 3.0 kb, which is consistent with a monocistronic transcript. *sigE* transcript levels were decreased at 30 and 48 h after nitrogen step-down. Several attempts to map the 5' ends of *sigE* transcripts using rapid amplification of 5' cDNA ends (RACE) were unsuccessful, possibly because the transcripts were unstable.

RNA-Seq data from total RNA samples obtained at 0, 6, 12, and 21 h after nitrogen step-down were consistent with the Northern blot data, with RPKM (reads per kilobase of coding sequence [CDS] model per million mapped reads) values of 9.08, 8.90, 20.51, and 24.86, respectively (B. Flaherty, submitted for publication). The RNA-Seq data did not show a clear 5' end for *sigE* transcripts, which is consistent with our RACE results. At all time points, staggered overlapping reads extended to about nucleotide -330 upstream of the first base of the open reading frame (ORF), with a gap in reads between about -60 and -140.

NtcA interacts with the upstream region of *sigE* in vitro. It has been shown that some heterocyst-specific promoters carry an NtcA binding site with a consensus sequence of tGTAN$_8$TACa, which is often located approximately 22 nucleotides upstream from a -10 TAN$_3$T box (12, 21). Our bioinformatics analysis of the *sigE* promoter region identified a canonical NtcA binding site 22 nucleotides upstream of a TAN$_3$T sequence, from position -683 to -698 relative to the *sigE* translational start site, which is in agreement with an independent analysis of NtcA binding sites in the *Anabaena* PCC 7120 genome (49). A second potential NtcA binding site that differs at one of the highly conserved bases is present at position -632 to -647. To determine if these sites interacted
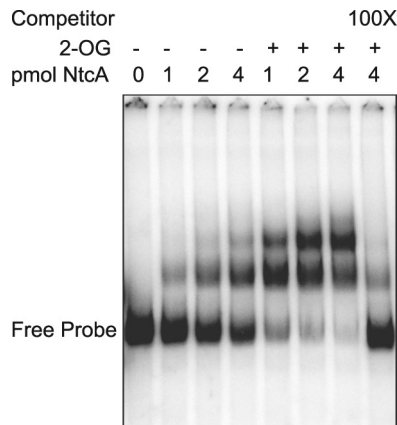
FIG. 2. NtcA binds to the *sigE* upstream intergenic region. Binding of purified 6× His-tagged NtcA to a 207-bp fragment from the intergenic region upstream of *sigE* was assayed by electrophoretic mobility shift assay (EMSA). Sample mixtures contained 0.1 fmol of labeled DNA fragment with or without 0.6 mM 2-oxoglutarate (2-OG). The last lane contains a 100-fold molar excess of the unlabeled probe as a competitor.

with NtcA, we performed electrophoretic mobility shift assays (EMSAs) using a radioactively labeled 207-bp PCR fragment containing both putative NtcA binding sites, purified 6× His-tagged NtcA protein, and the NtcA effector metabolite 2-oxoglutarate (2-OG). Figure 2 shows two band shifts of the labeled DNA fragment in the presence of increasing amounts of NtcA,

indicating NtcA binding to this region at two sites. The affinity of NtcA for this promoter fragment increased in the presence of 2-OG, and the binding was significantly reduced in the presence of unlabeled competitor DNA.

To determine if the highly conserved NtcA binding site is required for developmentally regulated expression of *sigE*, the site was mutated in plasmid pAM3652, which carries P_{sigE}-*gfp*. The NtcA binding site 5′-TGTA-N$_8$-TAC-3′ in the *sigE* promoter region was replaced with 5′-GTGC-N$_8$-TAC-3′, and the plasmid was introduced into wild-type *Anabaena* PCC 7120. The resulting strain, AMC1778, was analyzed by time-lapse microscopy after nitrogen step-down (data not shown). No significant differences in the pattern of GFP fluorescence were detected, showing that the conserved NtcA binding site is not essential for *sigE* expression *in vivo*, but this does not eliminate the possibility that the site contributes to normal expression.

To identify the minimal upstream region required for heterocyst-specific *sigE* expression, a deletion series of four fragments was used to drive *gfp* reporter expression (Fig. 3). Reporter plasmids containing $P_{sigE\text{-}P}$-*gfp* (−800 bp), $P_{sigE\text{-}P1}$-*gfp* (−500 bp), $P_{sigE\text{-}P2}$-*gfp* (−360 bp), $P_{sigE\text{-}P3}$-*gfp* (−260 bp), and $P_{sigE\text{-}P4}$-*gfp* (−79 bp) were transferred into wild-type *Anabaena* PCC 7120 to obtain reporter strains AMC1452, AMC1789, AMC1790, AMC1791, and AMC1792, respectively (Table 1). Observation of GFP fluorescence at 24 h after nitrogen step-down revealed that the strains containing $P_{sigE\text{-}P}$-*gfp*, $P_{sigE\text{-}P1}$-*gfp*, $P_{sigE\text{-}P2}$-*gfp*, and $P_{sigE\text{-}P3}$-*gfp* all showed higher levels of GFP fluorescence in heterocysts than in vegetative cells but that
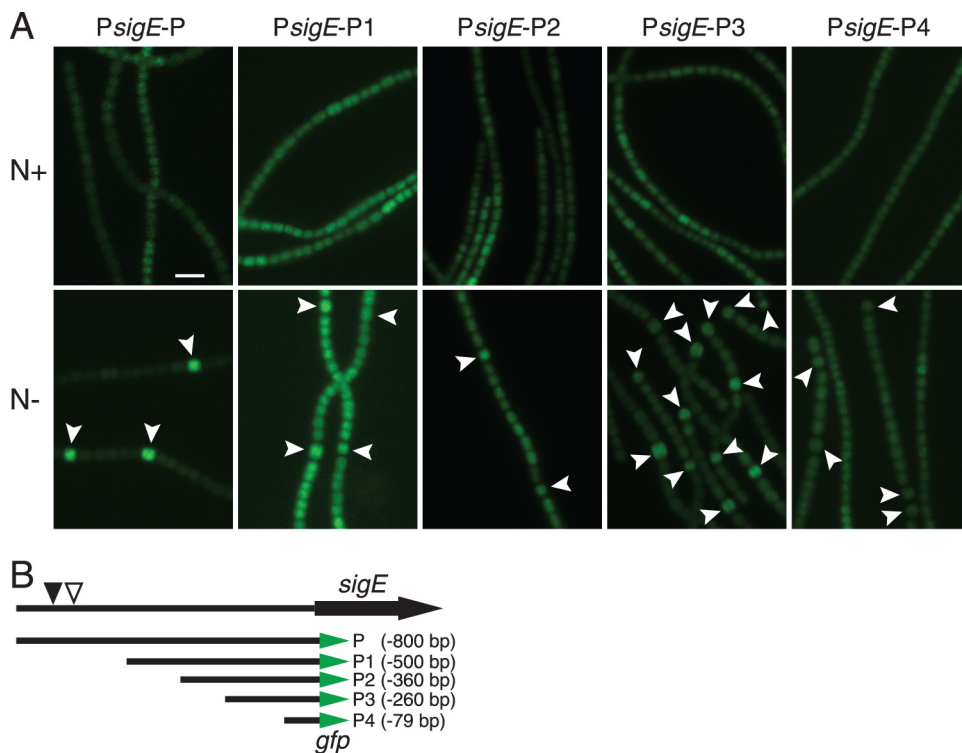

FIG. 3. GFP reporter expression from different upstream regions of *sigE*. (A) GFP fluorescence micrographs of filaments for reporter strains grown with (N+, upper panels) and without (N−, lower panels) combined nitrogen for 24 h. (B) Map showing upstream regions of *sigE* driving the *gfpmut2* reporter. Region P contains the entire 800-bp upstream intergenic region. The position of a conserved NtcA binding site is marked with a filled triangle, and a second potential binding site is marked with an open triangle. Heterocysts are indicated by arrowheads. Scale bar, 10 μm.
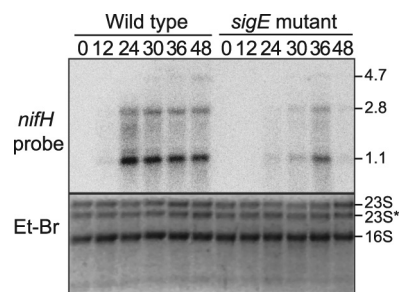
FIG. 4. Northern RNA blot analysis of *nifH* transcripts from the wild-type strain and *sigE* mutant strain AMC649 during heterocyst development. Lanes are labeled with hours after nitrogen step-down from BG-11 to BG-11$_0$ medium. The upper panel shows a Northern RNA blot probed with a radioactive probe for the *nifH* transcript. The lower panel shows an ethidium bromide-stained gel to show sample loading. Three transcripts containing *nifH* were detected at approximately 1.1 kb, 2.8 kb, and 4.7 kb.

P$_{sigE-P}$-*gfp*, which contained the entire upstream intergenic region, including the two NtcA binding sites, produced the strongest differential upregulation in heterocysts. The AMC1789 strain, containing P$_{sigE-P1}$-*gfp*, showed an overall higher level of GFP fluorescence for unknown reasons, but overall, expression was higher in heterocysts than in vegetative cells. The strain containing P$_{sigE-P4}$-*gfp* produced low-level expression in all cells. A strain carrying promoterless *gfp* on pAM1956 was dark. These data show that a 260-bp upstream region was sufficient to provide developmental regulation but that the entire upstream intergenic region, which includes the two NtcA binding sites, was required for robust regulation.

nifH expression is delayed and reduced in the *sigE* mutant strain. To determine if SigE is required for expression of genes in the later stages of heterocyst development, we analyzed the expression of the *nifH* gene, which encodes the nitrogenase iron protein, in wild-type and *sigE* mutant backgrounds. Total RNAs from the wild-type strain and the *sigE* mutant strain AMC649 were isolated at different time points after nitrogen step-down and then analyzed by Northern RNA blotting. Figure 4 shows the expression of *nifH* in the wild type and the *sigE* mutant when grown in medium containing nitrate (labeled 0) and at 12, 24, 30, 36, and 48 h after nitrogen step-down. The *nifH* probe hybridizes with three transcripts of approximately 1.1, 2.8, and 4.7 kb, which contain the *nifH*, *nifHD*, and *nifHDK* open reading frames, respectively (18). Transcripts containing *nifH* in the wild-type background were detected at low levels at 12 h after nitrogen step-down and reached their highest level by 24 h. In the *sigE* mutant strain AMC649, *nifH* transcripts were detected only weakly at 24 h and 30 h after nitrogen step-down, increased at 36 h but to lower levels than the wild-type strain, and significantly decreased at 48 h. These results show that expression of *nifH* is delayed and reduced in the *sigE* mutant and, together with data showing that *sigE* is upregulated at later times in proheterocysts, suggest that SigE is required for normal levels of *nifH* expression.

***sigE* influences the expression of a P$_{nifHD}$-*gfp* reporter during heterocyst development.** Time-lapse microscopy of heterocyst development was used to determine if *sigE* affects expression of a P$_{nifHD}$-*gfp* reporter and/or the heterocyst pattern *in vivo*. Strains containing a P$_{nifHD}$-*gfp* reporter integrated into
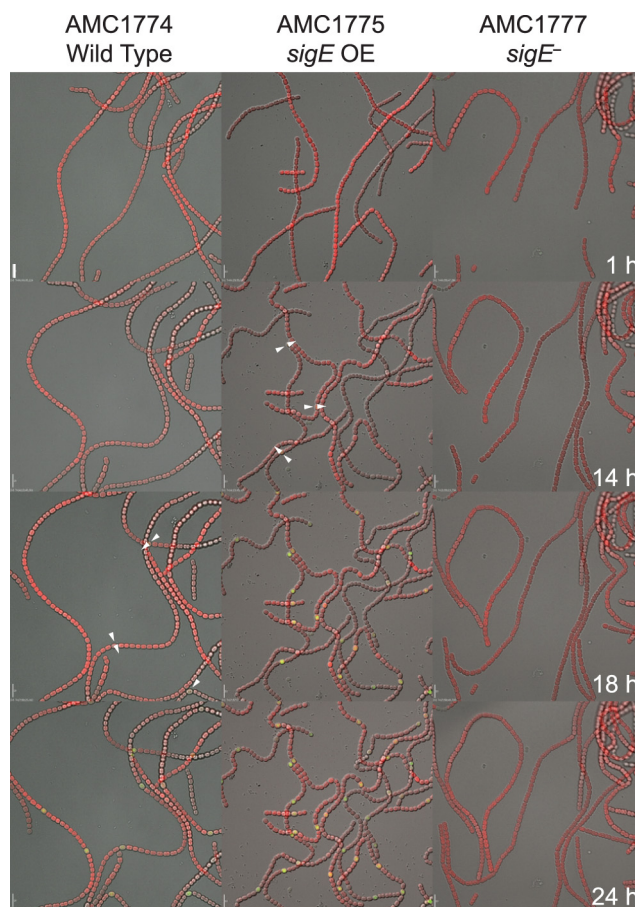

FIG. 5. Time-lapse microscopy images of a *nifHD-gfp* reporter construct integrated into the *nifH* locus on the chromosome in wild-type (AMC1774), *sigE* overexpression (OE) (AMC1775), and *sigE* mutant (AMC1777) backgrounds. Images are shown for 1, 14, 18, and 24 h after nitrogen step-down. Images are merged DIC (grayscale), autofluorescence (red), and GFP reporter fluorescence (green). Arrows indicate weak GFP fluorescence from proheterocysts. Scale bars, 10 μm.

the *Anabaena* PCC 7120 chromosome at the *nifH* locus were used for these studies. The P$_{nifHD}$-*gfp* reporter strain was constructed and characterized by K. Kumar and will be published elsewhere; the strain shows normal diazotrophic growth, and the integrated plasmid contains the chromosome region from the start of the *nifD* ORF to the end of the *nifS* ORF. The P$_{nifHD}$-*gfp* reporter was placed into three genetic backgrounds: wild type (AMC1774), a *sigE* knockout mutant (AMC1777), and a *sigE* overexpression strain (AMC1775). Heterocyst development was induced, and DIC, autofluorescence, and GFP fluorescence images were acquired every 15 min for 24 h for strains AMC1774 and AMC1775 and for 42 h for strain AMC1777. Figure 5 contains images corresponding to the indicated time points that were extracted from the time-lapse series (see Movies S1, S2, and S3 in the supplemental material).

At 1 h after nitrogen step-down, no GFP fluorescence or heterocyst morphological characteristics were evident in any of the three strains (AMC1774, AMC1775, and AMC1777). By 14 h, when morphological differentiation of proheterocysts was
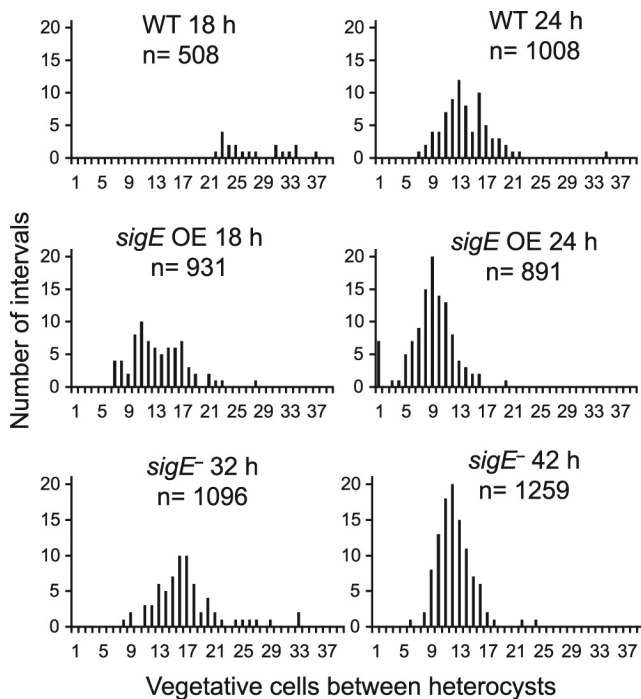
FIG. 6. The heterocyst pattern is influenced by *sigE*. The heterocyst pattern was determined for the strains shown in Fig. 5, which have a *nifHD-gfp* reporter in wild-type (AMC1774), *sigE* overexpression (AMC1775), and *sigE* mutant (AMC1777) backgrounds. For the wild-type (WT) and *sigE* overexpression (OE) backgrounds, the heterocyst pattern was scored by observing cell morphology and GFP fluorescence at 18 and 24 h after nitrogen step-down. For the *sigE* mutant background, which had delayed development and very low levels of *nifHD-gfp* reporter expression, the heterocyst pattern was scored by cell morphology at 32 and 42 h after nitrogen step-down. For each sample, the number of vegetative cells between heterocysts was scored, with adjacent heterocysts scored as an interval of zero. The *x* axis shows the number of intervals scored for each interval length. The average number of vegetative cells between heterocysts was 26.6 at 18 h and 13.1 at 24 h for the wild type, 12.4 at 18 h and 7.9 at 24 h for the *sigE* overexpression strain, and 15.8 at 32 h and 11.3 at 42 h for the *sigE* mutant.

not yet obvious in the wild type, the *sigE* overexpression strain (AMC1775) showed GFP fluorescence in individual cells that were morphologically distinguishable as proheterocysts. AMC1774 and AMC1777 filaments did not show any GFP fluorescence or proheterocysts at 14 h after nitrogen step-down.

At 18 h after nitrogen step-down, the wild-type strain (AMC1774) showed GFP fluorescence in some individual cells, which is somewhat delayed compared to expression of *nifH* determined by Northern RNA blotting experiments (Fig. 4); this is likely due to differences in growth conditions between batch cultures and samples on the microscope and the time required to accumulate sufficient GFP to be detectable under our time-lapse microscopy conditions, which use low excitation intensity to avoid photobleaching or cell damage. For the wild-type strain at 18 h, slight morphological changes in the cells that showed GFP fluorescence were apparent, and the average number of vegetative cells in the interval between heterocysts or GFP-bright proheterocysts was 26.6 (Fig. 6). The *sigE* overexpression strain showed strong GFP fluorescence at 18 h and

showed clear heterocyst morphological differentiation with an average interval of 12.4. In the *sigE* mutant strain (AMC1777), no GFP fluorescence or morphological differentiation was detected at 18 h after heterocyst induction.

At 24 h after nitrogen step-down, the P*nifHD*-*gfp* reporter in a wild-type background (AMC1774) showed GFP fluorescence in individual differentiated cells (Fig. 5) with an interval length of 13.1 vegetative cells (7.1% heterocysts) (Fig. 6). The *sigE* overexpression strain (AMC1775) showed a higher frequency of GFP-fluorescent heterocysts with an interval length of only 7.9 (11.2% heterocysts). These data show that extra copies of *sigE* caused accelerated heterocyst formation and expression of *nifH* and an increase in heterocyst frequency. At 24 h after nitrogen step-down, the *sigE* knockout mutant strain (AMC1777) did not show detectable GFP fluorescence or heterocyst morphological changes. By 32 h after nitrogen step-down, the *sigE* mutant showed morphological differentiation of some individual cells and an interval length of 15.8 (6.0% heterocysts), but no GFP fluorescence was evident. At 42 h, the *sigE* mutant had a pattern of mature heterocysts similar to that of the wild type (57) with an average interval length of 11.3 (8.1% heterocysts) (Fig. 6), but no GFP fluorescence was detectable even at this time.

The *sigE* gene is required for expression of other heterocyst-specific genes. Previous observations of unsynchronized cultures of a *sigE* mutant strain (AMC649) found little effect on heterocyst development or diazotrophic growth (27), and in our current experiments, we observed no significant difference in the growth rates of *sigE* mutant strains compared to the wild type once diazotrophic growth was established. However, the *sigE* expression data (Fig. 1), the slight delay in heterocyst development (Fig. 5 and 6), and the delayed and reduced *nifH* expression (Fig. 4) observed in the *sigE* mutant suggest that SigE may be required for transcription of genes expressed in the middle to late stages of heterocyst differentiation. To determine if SigE is required for the expression of other heterocyst-specific genes, we used qRT-PCR to examine the expression levels of genes that are upregulated during middle and late stages of heterocyst development. We selected *nifH*, *fdxH* (encoding a heterocyst-specific ferredoxin), and *hupL* (encoding the large subunit of uptake hydrogenase) as genes that are expressed during late stages of heterocyst development (9, 52). For a gene expressed during the middle stages of heterocyst development, we selected *hglE2* (all1646), which encodes a type I polyketide synthase potentially involved in heterocyst glycolipid synthesis (8, 15). For a baseline control mRNA, the expression of the housekeeping gene *rpoA*, which encodes the RNA polymerase alpha subunit, was analyzed by qRT-PCR during heterocyst development. *rpoA* message levels remained essentially invariant and at relatively low levels at all time points (Fig. 7); therefore, we used the *rpoA* message as an internal baseline control for our qRT-PCR experiments.

Figure 7 shows the transcript levels of *nifH*, *fdxH*, *hupL*, and *hglE2* relative to *rpoA* in wild-type and *sigE* mutant backgrounds during heterocyst induction. In medium containing nitrogen (0 h), *nifH* transcripts were not detected in either the wild type or the *sigE* mutant. At 12 h after nitrogen step-down, small amounts of *nifH* transcript were detected, but only in the wild-type strain. At 24 h a peak of *nifH* transcript was observed in the wild-type strain, while only trace amounts were de-
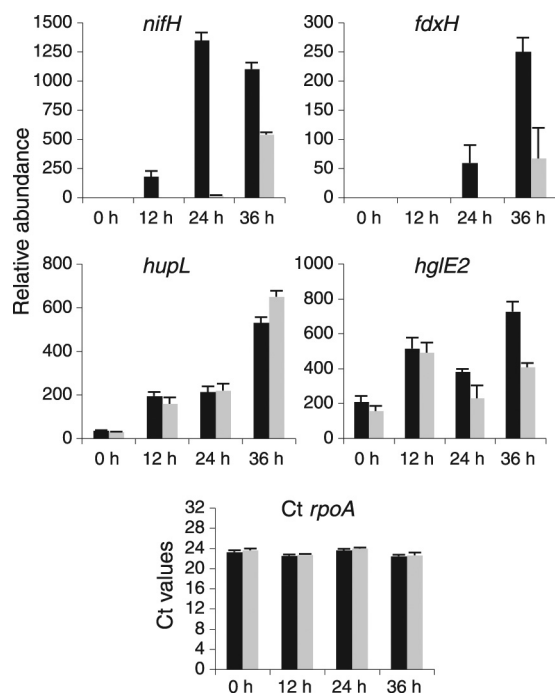
FIG. 7. Quantitative reverse transcription-PCR (qRT-PCR) measurements of mRNA abundance in wild-type and *sigE* mutant strains. Relative mRNA abundance was determined for *nifH*, *fdxH*, *hupL*, and *hglE2* using total RNA from wild-type *Anabaena* PCC 7120 (black bars) and the *sigE* mutant AMC649 (gray bars) at four time points after nitrogen step-down. The data represent average values and standard error measurements from three technical replicates, shown as percent relative abundance normalized to the RNA polymerase alpha subunit (*rpoA*) gene. The C_T *rpoA* graph shows the average C_T values for *rpoA* normalized against total RNA for wild-type *Anabaena* PCC 7120 and the *sigE* mutant AMC649.

tected in the *sigE* mutant strain. By 36 h, the levels of *nifH* transcript in the *sigE* mutant strain had increased to about half the wild-type transcript level. These results are in accordance with the data obtained by Northern RNA blot analysis (Fig. 4) and with the P_{nifHD}-*gfp* reporter, which supports the hypothesis that SigE regulates the expression of *nifH*.

Expression of the *fdxH* gene was similar to that of *nifH* although somewhat delayed (Fig. 7). Message was first detected for wild-type filaments at 24 h and was abundant by 36 h, but for the *sigE* mutant strain, *fdxH* message was detectable at low levels in only the 36-h sample. These data suggest that SigE regulates the expression of *fdxH* during heterocyst development.

Although previous studies with *Anabaena* PCC 7120 showed expression of *hupL* only during the late stages of heterocyst development (9), *hupL* transcripts have been detected in ammonium-grown vegetative cells of *Anabaena variabilis* (5). Our present data show very low levels of *hupL* transcripts in samples from vegetative cells in nitrate-containing medium (0 h) and increasing levels at later stages of heterocyst development (Fig. 7). The *hupL* transcript levels were not significantly different for the *sigE* mutant strain and the wild type, indicating that SigE is not involved in the regulation of the *hupSL* operon.

For the *hglE2* gene, transcripts were present in all samples,

with the highest levels at 12 h and 36 h after heterocyst induction and a decrease at 24 h (Fig. 7). The *hglE2* transcripts in the *sigE* mutant were significantly lower than those in the wild type at 24 and 36 h, with the levels at 36 h about half the wild-type levels. These data suggest that SigE may regulate *hglE2* expression at late stages of heterocyst development but not in vegetative cells or at early stages of development. Together, these data indicate that SigE is involved with the regulation of some but not all genes at late stages of heterocyst development.

DISCUSSION

Previous studies had found that none of the *Anabaena* PCC 7120 group 2 sigma factors *sigB*, *sigB2*, *sigC*, *sigD*, and *sigE* is individually essential for heterocyst development or diazotrophic growth (6, 27). However, more recent results showed that *sigC* and *sigE* are upregulated in differentiating heterocysts (3). In this work, using a more detailed examination of gene expression and heterocyst development, we show that the *sigE* gene is required for normal heterocyst development and for normal expression of certain heterocyst genes.

Expression analysis of *sigE* by Northern RNA blotting showed developmental upregulation of transcripts beginning 12 h after nitrogen step-down, which is consistent with our previous observations using a P_{sigE}-*gfp* reporter (3). The *sigE* transcripts showed significant degradation, and attempts to identify a specific *sigE* transcriptional start site were not successful, indicating a relatively short half-life, which is normal for most bacterial mRNAs.

Bioinformatic analysis identified a putative consensus NtcA binding site in the *sigE* upstream region, which could contribute to its developmental regulation. EMSA showed that NtcA binds to a DNA fragment containing this site and produced two shifted bands, indicating a second binding site. Binding was enhanced by the presence of 2-OG. Further analysis of the fragment sequence allowed the identification of another potential binding site that differs from the consensus at one position. Deletion of both binding sites resulted in a partial loss of developmental expression, indicating that NtcA plays a partial role in the regulation of *sigE*. Because *ntcA* mutants are defective for heterocyst development and highly pleiotropic, it is not possible to use *ntcA* mutants to establish a direct role for NtcA in the regulation of heterocyst-specific genes, especially those upregulated at the middle to late stages of development. Many *Anabaena* genes show complex regulation by multiple promoters, and it is likely that *sigE* expression is regulated by multiple transcription factors to provide for expression in vegetative cells and upregulation in differentiating cells (11, 12, 34). Further biochemical studies will be required to determine the function of the two NtcA binding sites and if they interact with each other or alter DNA topology to influence the expression of *sigE* or the adjacent gene *all4248*.

Because *sigE* is expressed during the later stages of heterocyst development, we hypothesized that *sigE* may be involved in the expression of the nitrogen fixation (*nif*) genes. The regulation of the *Anabaena* PCC 7120 *nifHDK* operon has been studied for over 25 years (14, 18, 20, 43, 44, 50, 53); however, little is known about the transcription components that regulate this operon. Our results suggest that *sigE* is in-

volved in *nifH* expression. However, regulated *nifH* expression was not completely abolished, which means that other transcription factors must be involved and that sigma factors other than SigE must be able to provide transcription initiation. It is not clear if the residual expression is part of normal regulation or a consequence of cross talk among the multiple group 2 sigma factors in *Anabaena* PCC 7120. Demonstrating direct regulation of *nifH* expression by SigE will require *in vitro* transcription assays using purified components. However, these experiments are especially challenging because the *cis*-acting sequences that constitute the *nifH* promoter have not been identified in *Anabaena* PCC 7120, and recent data with *Anabaena variabilis* suggest that multiple distant promoters and RNA-processing events may be involved in expression of the *nifHDK* operon (50).

In addition to the delayed and reduced expression of *nifH*, the *sigE* mutant showed delayed heterocyst morphological differentiation; mature heterocysts were formed, but not until 32 h after nitrogen step-down (about 14 h longer than for the wild-type strain). This indicates that the SigE regulon includes genes required for normal heterocyst differentiation and morphogenesis but also that there must be some redundancy in the regulation. This redundancy may be a selective advantage, because it would provide robustness to the developmental process.

Overexpression of *sigE* caused premature expression of P*nifHD*-*gfp* and a higher heterocyst frequency than normal. The expression of P*nifHD*-*gfp* in the *sigE* overexpression strain is first detectable at 14 h after heterocyst induction (4 hours earlier than for the wild-type strain). Therefore, *sigE* is both necessary for normal *nifHDK* expression and sufficient to cause precocious expression of the operon. The simplest scenario is that SigE interacts directly with a promoter for the *nifHDK* operon; however, it is possible that the regulation is indirect. In addition, heterocyst morphogenesis and maturation occurred earlier and the final heterocyst frequency was higher in the *sigE* overexpression strain, indicating that the SigE regulon includes genes that influence the progression and timing of heterocyst differentiation.

Because *sigE* influenced the expression of *nifH*, we hypothesized that *sigE* might also regulate the expression of other late-stage heterocyst-specific genes such as *fdxH* and *hupL* (9, 52). qRT-PCR revealed that the expression levels of *nifH* and *fdxH* were both reduced in the *sigE* mutant strain. On the other hand, the expression of *hupL* was upregulated normally in the *sigE* mutant after nitrogen step-down. These results show that *sigE* is required for the expression of only a subset of late-stage heterocyst-specific genes and indicate that at least some factors required for *hupL* expression are different from those required for *nifH* and *fdxH* expression.

The *hglE2* gene is necessary for the heterocyst glycolipid layer and is developmentally upregulated during the middle stage of heterocyst differentiation (1, 15, 55). In the *sigE* mutant strain, the *hglE2* expression levels at 12 h were similar to those in the wild-type strain; however, by 36 h after nitrogen step-down, the transcript levels of *hglE2* were only half of the wild-type levels. These data suggest that *hglE2* is expressed in at least two ways: expression at early stages of heterocyst development that is SigE independent and SigE-dependent expression at a later stage.

The focus of this work was on gene regulation related to heterocyst development and nitrogen fixation, but it seems likely that SigE in *Anabaena* PCC 7120 could also be involved in the regulation of sugar catabolic genes, as has been demonstrated for other cyanobacteria (24, 38). Although *gfp* reporter experiments showed that the *sigE* gene was upregulated in differentiating cells after nitrogen step-down, it was also expressed at a low level in uninduced filaments and in the vegetative cells of induced filaments, which is consistent with SigE being involved in gene expression in those cells.

The results presented in this work indicate that SigE is involved in the expression of heterocyst-specific genes that are upregulated during the later stages of development. A *sigE* mutant, similarly to other cyanobacterial sigma factor mutants, showed incomplete genetic penetrance and expressivity. This has made the study of sigma factor function in cyanobacteria difficult. However, observations of quantitative changes in cellular and molecular phenotypes are beginning to tease out the roles played by sigma factors in cyanobacterial gene expression.

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