

Temporal and Spatial Regulation of the Four Transcription Start Sites of *hetR* from *Anabaena* sp. Strain PCC 7120[∇]

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The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 forms nitrogen-fixing heterocysts in a periodic pattern in response to combined-nitrogen limitation in the environment. The master regulator of heterocyst differentiation, *HetR*, is necessary for both pattern formation and commitment of approximately every 10th cell of a filament to differentiation into a heterocyst. In this study, the individual contributions of four transcriptional start points (tsps) in regulation of transcription of *hetR* were assessed, and the effects of the regulatory genes *patS*, *hetN*, and *patA* on transcription from the tsps were determined. The tsp located at nucleotide -271 relative to the translational start site (-271 tsp) was the most tightly regulated tsp, and fluorescence from a -271 tsp-green fluorescent protein (GFP) reporter fusion was observed initially in groups of two cells and later in single cells arranged in a spatial pattern that mimicked the pattern of heterocysts that emerged. Conversely, the fluorescence from the -184 and $-728/-696$ tsp-GFP reporter fusions was uniform throughout filaments. Transcription from the -271 tsp was severely downregulated in a strain in which the *patA* gene, which encodes a positive regulator of differentiation, was deleted, and it was not detectable in strains overexpressing the genes encoding the negative regulators of differentiation, *patS* and *hetN*. In strains lacking the -271 tsp of *hetR*, pattern formation, the timing of commitment to differentiation, and the number of cells that differentiated into heterocysts were affected. Taken together, these results demonstrate the role of regulation of the -271 tsp of *hetR* in the genetic network that governs heterocyst pattern formation and differentiation.

The filamentous cyanobacterium *Anabaena* sp. PCC 7120 fixes dinitrogen in specialized cells called heterocysts. When the levels of fixed nitrogen are sufficient to support robust growth, the filaments are comprised entirely of undifferentiated vegetative cells. However, when the levels of fixed nitrogen become limiting (nitrogen starvation), certain cells of the filament develop into terminally differentiated heterocysts, where nitrogen fixation takes place. Heterocysts provide a microoxic environment for the oxygen-labile enzyme nitrogenase, which catalyzes the formation of ammonia from dinitrogen. The product of nitrogen fixation is transported from a heterocyst to the adjacent vegetative cells in the form of glutamine (18), and the heterocyst in return receives fixed carbon (28). Thus, the two cell types are metabolically interdependent and have different roles in the filament.

Approximately every 10th cell in a filament differentiates into a heterocyst to create a periodic pattern consisting of two cell types. Heterocysts do not divide, and the distance between two heterocysts increases as the intervening vegetative cells multiply. As the distance becomes greater, a cell approximately midway between two heterocysts differentiates, and thus the heterocyst pattern is maintained (12). This one-dimensional pattern of two cell types is one of the simplest and oldest patterns elaborated by a multicellular organism (27), making it an ideal system for investigation of developmental regulation

in prokaryotes and definition of the minimal requirements for patterning and morphogenesis (22).

In *Anabaena* sp. PCC 7120 nitrogen starvation results in accumulation of 2-oxoglutarate because this strain lacks 2-oxoglutarate dehydrogenase (25). 2-Oxoglutarate is thought to bind to the global nitrogen regulator NtcA and enhances its capacity as a transcriptional regulator (15). NtcA in turn activates a range of genes involved in heterocyst formation and function (11). One of these genes is *hetR*. *HetR* is considered the master regulator of heterocyst differentiation. The level of expression of *hetR* in a cell correlates with the cell's developmental fate. Induction of expression of *hetR* is sufficient to trigger the differentiation process, even in the presence fixed nitrogen (5), and heterocyst formation does not take place in its absence (4). *HetR* positively autoregulates itself at the level of transcription (1). The *HetR* protein binds to its own promoter and to the promoters of other heterocyst-specific genes, suggesting that it functions as a regulator of transcription (13).

Transcription of *hetR* is both temporally and spatially regulated. Northern blot experiments have shown that *hetR* transcription levels begin to increase by 0.5 h after nitrogen starvation (5). Between 6 and 18 h during induction of heterocyst differentiation, there is up to a 5-fold increase in transcript levels (4). *HetR* also shows spatial patterning of transcription. In a filament of undifferentiated vegetative cells grown in the presence of combined nitrogen, a low level of transcription is observed for all of the cells of the filament (1). In contrast, by 3.5 h after removal of combined nitrogen there is patterned upregulation of transcription in groups of cells in the filament (1), and by 6 to 8 h transcription is localized to cells that are arranged in a periodic pattern and will differentiate into het-

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erocysts (1, 6). Subsequently, upregulation of *hetR* transcription is observed only in cells that are midway between two heterocysts and will presumably also differentiate to preserve the pattern of heterocysts in a growing filament (1).

PatS is a negative regulator of heterocyst differentiation that is thought to mediate pattern formation by suppression of differentiation of neighboring cells by a source cell. Its C-terminal pentapeptide, RGSGR, has been shown to inhibit heterocyst differentiation (23, 30) and also prevent HetR from binding to DNA in the promoter region of *hetR* (13). HetN is another regulator of heterocyst differentiation that is thought to mediate pattern maintenance in a similar fashion. Heterocyst differentiation and the patterned expression of *hetR* are disrupted by overexpression of *hetN* (6). HetN and PatS function independently, although they converge functionally at or before *hetR* in the regulatory network controlling differentiation (2, 14). PatA is a positive regulator of differentiation that downregulates the inhibitory effects of PatS and HetN and promotes differentiation independent of its effects on PatS and HetN (2, 17). PatS, HetN, and PatA have each been shown to impact the spatial and temporal expression of *hetR* (5, 6, 13, 16).

The promoter region of *hetR* is complex. Four transcriptional start points (tsp) are located at nucleotides -728 (-728 tsp), -696 , -271 , and -184 relative to the translational start site, and each tsp gives rise to a distinct mRNA molecule (5). The transcript originating at the -184 tsp is present regardless of the nitrogen status (5, 19). Transcription from tps -728 and -696 is induced upon nitrogen starvation (5). NtcA is required for transcription from tsp -728 (19), and its activity is mediated through NrrA, a response regulator of the OmpR family (7, 8). Transcription from tsp -271 is also induced upon nitrogen starvation. A functional copy of HetR is essential for transcription from tsp -271 (5). The HetR protein binds to a region of DNA around the -271 tsp (13), and this transcription start point is thought to be involved in positive autoregulation (5).

Differential gene expression has long been recognized as a factor that has an important role in regulating heterocyst differentiation (29). We investigated the temporal and spatial transcription patterns of the four transcription start sites of *hetR* and found that only the -271 tsp is regulated spatially. We also analyzed the role played by the heterocyst regulators PatA, PatS, and HetN in controlling transcription and found that each regulator affects transcription from a subset of tps.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Anabaena* sp. PCC 7120 (wild type) and its derivatives are described in Table 1. The growth conditions used and regulation of expression of the *petE* promoter (P_{petE}) have been described previously (22). For analysis of the temporal transcription profile of *hetR*, *Anabaena* strains were grown in BG-11 medium buffered with 30 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (pH 8.0) and supplemented with 10 mM NH_4Cl and 5 mM NaHCO_3 . Cells were washed twice in medium lacking a fixed-nitrogen source (BG-11₀) and transferred to BG-11₀. In all other experiments, *Anabaena* strains were grown in BG-11 medium buffered with 10 mM HEPES (pH 8.0) and supplemented with 2 mM $(\text{NH}_4)_2\text{SO}_4$ and 5 mM NaHCO_3 prior to transfer to BG-11₀, when appropriate. *Escherichia coli* strains bearing plasmids were grown in medium supplemented with 50 $\mu\text{g/ml}$ kanamycin sulfate or 100 $\mu\text{g/ml}$ spectinomycin sulfate. Strains of *Anabaena* bearing plasmids were grown in medium supplemented with 45 $\mu\text{g/ml}$ neomycin sulfate, and strains of *Anabaena* bearing suicide plasmids recombined into the chromosome were

grown in medium supplemented with 2.5 $\mu\text{g/ml}$ spectinomycin sulfate and streptomycin sulfate. Bright-field microscopy and fluorescence microscopy were performed as previously described (2). All fluorescence images were captured using the same exposure and sensitivity camera settings. Plasmids were introduced into different strains of *Anabaena* by conjugation as previously described (9).

Construction of strains and plasmids. Strains, plasmids, and primers are listed in Table 1. PCR-derived constructs were sequenced to verify their integrity. For creation of tsp-reporter fusions, the promoter region of *hetR* was separated into five regions bearing one or more transcriptional start points of *hetR*. The different tsp regions were cloned in front of the coding regions of either *gfp* (encoding green fluorescent protein [GFP]) or *lacZ*. The nucleotide boundaries of the tsp regions for each fusion are shown in Fig. 1.

The tsp-*gfp* fusions were generated by inserting the appropriate tsp regions into plasmid pAM1956 (30) as SacI-KpnI fragments. The plasmids that contained the *gfp* fusions and the primers (in parentheses) used to generate the corresponding regions of the *hetR* promoter are as follows: pRR139 for the $-728/-696$ tsp-*gfp* fusion (Sac GFP L-F3 and Kpn GFP-L-R2); pRR149 for the $-728/-696(+132)$ tsp-*gfp* fusion [the $-728/-696(+132)$ tsp fusion includes an additional 132 bases of the *hetR* promoter downstream of those in the initial construct] (Sac GFPL-F3 and Kpn GFP L R3); pRR140 for the -271 tsp-*gfp* fusion (Sac GFP-S-F and Kpn GFP-S-R2); pRR151 for the -184 tsp-*gfp* fusion (Sac GFP C-F and Kpn GFP-M-R3); and pRR150 for the $-271/-184$ tsp-*gfp* fusion (Sac GFP-S-F3 and Kpn GFP-M-R3). The tsp-*lacZ* fusions were generated by inserting the appropriate tsp region into plasmid pNC101 (22) as a BamHI-SmaI fragment. The plasmids that contained the *lacZ* fusions and the primers (in parentheses) used to generate the corresponding regions of the *hetR* promoter are as follows: pRR142 for the $-728/-696$ tsp-*lacZ* fusion (lacZ Bam 696 Fwd and lacZ Sma 696 Rev2); pRR152 for the $-728/-696(+132)$ tsp-*lacZ* fusion (lacZ Bam 696 Fwd and lacZ Sma 696 R3); and pRR143 for the -271 tsp-*lacZ* fusion (lacZ Bam 271 Fwd and lacZ Sma 271 R2).

Plasmid pDR211 is a mobilizable shuttle vector derived from pAM504 (26) and contains the *patS* gene under the control of a copper-inducible promoter (P_{petE}). Primers Xho-Bam-PpetE-F and PpetE-Nde-Pst-R were used to amplify P_{petE} , and the resultant DNA fragment was cloned into pGEM-T (Promega). P_{petE} was subsequently moved into pBluescript SK(+) (Stratagene) as an XhoI-PstI fragment. The *patS* gene was then inserted downstream of P_{petE} to generate $P_{petE-patS}$. The *patS* gene was amplified using primers patS-NdeI-F and patS-SalI-SpeI-R, and the resultant DNA fragment was cloned into pGEM-T before it was moved as an NdeI-SpeI fragment behind P_{petE} in pBluescript. $P_{petE-patS}$ was then moved into pAM504 as a BamHI-SacI fragment to obtain plasmid pDR211.

Plasmid pDR325 is a suicide vector derived from pRL277 (1) and contains a 3,605-bp fragment starting 1,691 bp upstream of the *hetR* coding region and ending 1,014 bp downstream of the *hetR* coding region. This 3,605-bp fragment was amplified by PCR using PCC 7120 chromosomal DNA as the template with primers hetR5'for and hetR-Chr-SacI-R and was cloned into pRL277 as a BglII-SacI fragment using restriction sites introduced into the primers to create pDR325. The *hetR* promoter and coding region in pDR325 was replaced with one of two versions lacking either nucleotides -295 to -268 or nucleotides -317 to -268 using NcoI and SpeI to obtain plasmids pRR164 and pRR165, respectively. The segment of DNA bearing the deletions was generated by overlap extension PCR using primers PhetR-BamHI-F, 271 tsp Rev, 271 tsp Fwd, and hetR-SacI-SpeI-R to create pRR164 and using primers PhetR-BamHI-F, Rev-N, Fwd-N, and hetR-SacI-SpeI-R to create pRR165. Strains UHM156 and UHM157 lack nucleotides -295 to -268 and nucleotides -317 to -268 of the *hetR* promoter, respectively, and were created by allelic replacement as previously described (20) using plasmids pRR164 and pRR165, respectively.

RNA isolation and analysis. Cells grown for RNA isolation were flash-frozen in liquid nitrogen at specified time intervals and stored at -80°C until they were processed. TRIzol (Invitrogen) was used for RNA extraction as previously described (22). Three micrograms of RNA was used for rapid amplification of cDNA ends (RACE) as previously described (22). Primers designed to bind downstream of the -184 tsp were used to amplify the -271 and -184 transcripts (primers 184new1 and new184nesrev for cDNA synthesis and RACE, respectively), and primers designed to bind downstream of nucleotide -696 were used to amplify the -728 and -696 transcripts (primers 696new1 and 696new2 for cDNA synthesis and RACE, respectively). RACE products were sequenced to verify their integrity. Ten micrograms of RNA was used for Northern blotting as previously described (5). Primers hetRcf-NdeI and hetRcr-SacI were used to make the *hetR*-specific probe. Northern blot and RACE experiments were done two or more times.

TABLE 1. Strains, plasmids, and PCR primers used in this study

Strain, plasmid, or primer	Relevant characteristics	Source or reference
<i>Anabaena</i> sp. strains		
PCC 7120	Wild type	Pasteur Culture Collection
UHM101	$\Delta patA$	20
120PN	$P_{petE}-hetN$	6
UHM103	$\Delta hetR$	2
HM156	Nucleotides -295 to -268 of promoter of <i>hetR</i> deleted	This study
UHM157	Nucleotides -317 to -268 of promoter of <i>hetR</i> deleted	This study
Plasmids		
pNC101	Shuttle vector derived from pAM504 for replication in <i>E. coli</i> and <i>Anabaena</i> with promoterless <i>lacZ</i> gene; Km ^r Nm ^r	22
pAM504	Shuttle vector for replication in <i>E. coli</i> and <i>Anabaena</i> ; Km ^r Nm ^r	26
pAM1956	Shuttle vector for replication in <i>E. coli</i> and <i>Anabaena</i> with promoterless <i>gfp</i> gene; Km ^r Nm ^r	30
pAM1951	Shuttle vector with $P_{patS}-gfp$; Km ^r Nm ^r	30
pSMC127	pAM504 carrying $P_{hetR}-gfp$	6
pDR211	Shuttle vector derived from pAM504 with $P_{petE}-patS$	This study
pRR139	pAM1956 with -728/-696 <i>tsp</i> region fused to <i>gfp</i>	This study
pRR149	pAM1956 with -728/-696(+132) <i>tsp</i> region fused to <i>gfp</i>	This study
pRR140	pAM1956 with -271 <i>tsp</i> region fused to <i>gfp</i>	This study
pRR150	pAM1956 with -271/-184 <i>tsp</i> region fused to <i>gfp</i>	This study
pRR151	pAM1956 with -184 <i>tsp</i> region fused to <i>gfp</i>	This study
pRR142	pNC101 with -728/-696 <i>tsp</i> region fused to <i>lacZ</i>	This study
pRR143	pNC101 with -271 <i>tsp</i> region fused to <i>lacZ</i>	This study
pRR152	pNC101 with -728/-696(+132) <i>tsp</i> region fused to <i>lacZ</i>	This study
pDR325	Suicide vector derived from pRL277 containing a 3,605-bp fragment starting 1,691 bp upstream and ending 1,014 bp downstream of the <i>hetR</i> coding region	This study
pRR164	Suicide vector derived from pDR325 lacking nucleotides -295 to -268 in the <i>hetR</i> promoter region	This study
pRR165	Suicide vector derived from pDR325 lacking nucleotides -317 to -268 in the <i>hetR</i> promoter region	This study
Primers (5'-3')		
hetR5' for	TTTAGATCTGCTGCTCGTTCTCAGCCACAGAGATTTGTCC	
hetR-Chr-SacI-R	ATATAGAGCTCATGTCTTGGCTCAGTCGCGGATGATGG	
Sac GFP L-F3	CACACGAGCTCGACAAAGGACTTATAATCAAAACAAATG	
Kpn GFP-L-R2	ATATGGTACCCTTAGCAATACCTTTTATTTCC	
Sac GFP-S-F3	ATTATGGAGCTCGGAAATAAAAGGTATTGCTAAGTTGG	
Kpn GFP-S-R2	ATATGGTACCCTACTGGCGAACTTTATGGTTC	
Sac GFP-C-F	TTATGAGTCCATAAAAGTTCGCCAGTAATTGACAAATG	
Kpn GFP M R3	AAATGGTACCATTACAAATAGTTGAATAGCACCG	
Kpn GFP L R3	ATATGGTACCCTCACTAACGCCATTGAATC	
lacZ Bam 696 Fwd	CACACGGATCCGACAAAGGACTTATAATCAAAACAAATG	
lacZ Sma 696 Rev2	ATATCCCGGGCTTAGCAATACCTTTTATTTCC	
lacZ Bam 271 Fwd	ATTATGGGATCCGAAATAAAAGGTATTGCTAAGTTGG	
lacZ Sma 271 R2	ATATCCCGGGTACTGGCGAACTTTATGGTTC	
lacZ Sma 696 R3	ATATCCCGGGCTCACTAACGCCATTGAATC	
696new2	AATCGCACAAATCTCTCCTTGAGGGTG	
696new1	TTTGCTCTTATGGCAGGTGATAGGTCG	
new184nesrev	CGCTTGATCAGATCGATGTCGTTACTC	
184new1	CATTGCACTGGGGCCAAGACGCTT	
hetRcf-NdeI	CATATGAGTAACGACATCGATCTGATC	
hetRcr-SacI	GAGCTCTTAATCTTCTTTTCTACCAAACACC	
Xho-Bam-PpetE-F	TATATCTCGAGGGATCCGCTGAGGTACTGAGTACACAGC	
PpetE-Nde-Pst-R	ATATACTGCAGCATATGGTTCCTAACCTGTAGTTTTATTTTC	
patS-NdeI-F	CATATGAAGGCAATTATGTTAGTGAATTTCTGTGATGAG	
patS-SalI-SpeI-R	ACTAGTCTCGACCTATCTACCACTACCGCCTCATCACAG	
271 <i>tsp</i> Fwd	CAGATAAGTTCGGATAATAGGGCCATAAAGTTCGCCAGTA	
271 <i>tsp</i> Rev	TACTGGCGAACTTTATGGCCCTATTATCCGGAACCTTATCTG	
Fwd-N	CAAATTTATTGACGCTATTAAGAGCCATAAAGTTCGCCAGTA	
Rev-N	TACTGGCGAACTTTATGGCTCTTTAATAGCGTCAATAAATTTG	
PhetR-BamHI-F	ATATAGGATCCAACCCTTATGACAAAGGAC	
hetR-SacI-SpeI-R	TATATAGAGCTCACTAGTACTTTTATTCACCTCTGGGTGC	

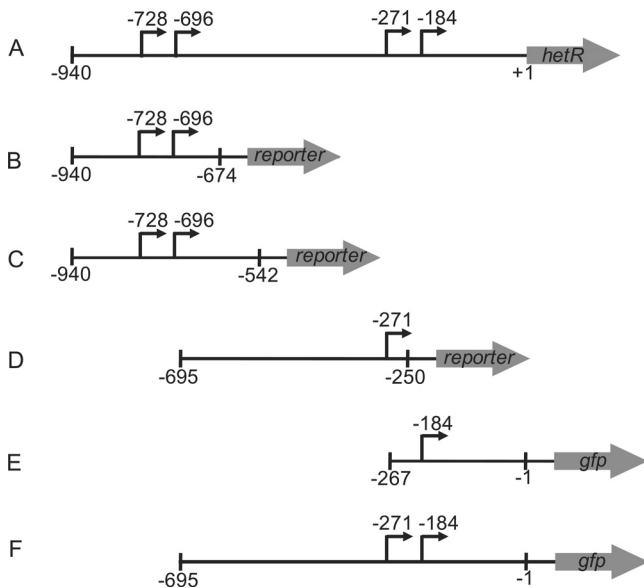


FIG. 1. Schematic diagrams of reporter fusions with tsp regions of the *hetR* promoter. (A) *hetR* promoter and coding region with its four transcriptional start points. (B) $-728/-696$ tsp-reporter fusion. (C) $-728/-696(+132)$ tsp-reporter fusion. (D) -271 tsp-reporter fusion. (E) -184 tsp-reporter fusion. (F) $-271/-184$ tsp-reporter fusion. The arrows indicate tsps, and the vertical lines indicate the boundaries of sequences from the promoter region of *hetR* that were included in each of the fusions. The numbers are positions relative to the translational start site of *hetR*.

β -Galactosidase assays. Cells were prepared and β -galactosidase activity was determined as previously described (22). Enzyme activity is expressed as nanomoles of *o*-nitrophenol per minute per milligram of protein.

RESULTS

Expression from the -271 tsp of *hetR* is tightly regulated.

Northern blotting and primer extension have been used to show that the levels of transcription from *hetR* tsp -184 are similar under nitrogen-replete and nitrogen starvation conditions, while expression from the *hetR* -728 , -696 , and -271 tsps is induced after deprivation of combined nitrogen (5, 19). Because transcription from tsp -271 has been implicated in direct positive autoregulation of *hetR*, which has the potential to rapidly amplify transcription of *hetR*, we were interested in examining transcription from each of the tsps using a more sensitive approach. To examine the tightness of regulation of transcription from the individual tsps, RACE, a very sensitive procedure, was used with RNA extracted from the wild type before induction of heterocyst differentiation and at 0.5, 1, 2, 3, 4, 5, and 6 h after induction of differentiation in BG-11₀, which lacks combined nitrogen. Under the conditions tested, transcription from tsps -728 , -696 , and -184 was detected at all eight time points (Fig. 2). However, the transcript originating from the -271 tsp was not detected in nitrogen-replete conditions or at 0.5 and 1 h. Transcription was detected for the -271 tsp 2 h after removal of combined nitrogen or later. A band for a third RACE product slightly smaller than the -271 tsp RACE product was observed at 0, 0.5, and 1 h (Fig. 2B). This band has been observed previously and was shown to correspond to the -184 tsp, suggesting that nonspecific prim-

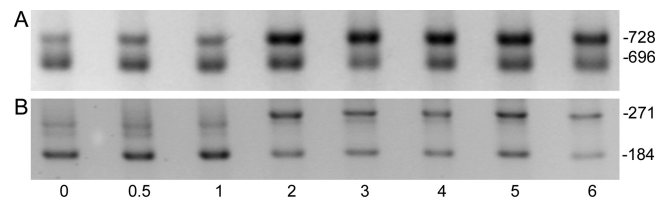


FIG. 2. Temporal patterns of transcription from the tsps of *hetR*. (A) RACE products corresponding to the -728 and -696 tsps. (B) RACE products corresponding to the -271 and -184 tsps. The times (in hours) after removal of combined nitrogen are indicated below the gels.

ing, not another tsp, is responsible for this product (22). Isolation and sequencing in this case indicated that the corresponding bands in Fig. 2B also corresponded to transcription from the -184 tsp. These results indicate that transcription originates from the -728 and -696 tsps even under nitrogen-replete conditions. Conversely, transcripts originating from the -271 tsp are not detectable prior to 2 h after nitrogen step-down, even with the ultrasensitive RACE assay, which includes PCR amplification of products.

Transcription from the -271 tsp is spatially regulated. To determine which of the transcriptional start points of *hetR* contribute to patterned expression of *hetR* and which transcriptional start points contribute to the uniform background expression in filaments, the promoter region of *hetR* was separated into five regions with one or more of the *hetR* transcriptional start points, each region was fused individually to *gfp*, and the resulting fusions were introduced into the wild-type strain using a replicating plasmid. The individual tsp regions and resulting fusions are shown in Fig. 1. Consistent with previous reports on the spatial regulation of expression of *hetR*, expression, as indicated with the full-length promoter of *hetR* fused to *gfp*, was observed in all cells, was induced in certain cells prior to differentiation, and continued in mature heterocysts (1, 6) (Fig. 3A). In contrast, the wild-type strain bearing the $-728/-696$ tsp-*gfp* fusion exhibited considerable fluorescence throughout the filament under both nitrogen-replete and nitrogen starvation conditions (Fig. 3B). There was no appreciable difference in fluorescence between vegetative cells, proheterocysts, and heterocysts. The GFP fluorescence from this fusion was considerably brighter than that from the full-length promoter-transcriptional fusion of *hetR* to *gfp*. Similar levels of fluorescence were also observed in vegetative cells, proheterocysts, and heterocysts of the wild-type strain with the -184 tsp fusion (Fig. 3C). There was not a notable difference in the levels of GFP fluorescence between nitrogen-replete and nitrogen starvation conditions.

The wild-type strain bearing the -271 tsp-*gfp* fusion showed spatially patterned transcription (Fig. 3D to H). Temporal and spatial aspects of transcription from this fusion were examined under nitrogen-replete conditions and at specific time points after induction of heterocyst differentiation. GFP fluorescence was barely detectable under nitrogen-replete conditions. Upon induction, fluorescence increased by 4 h in groups of cells, most of which were in pairs, and the intensity of fluorescence increased with time. Localization of fluorescence to primarily single cells was seen at 18 h after nitrogen step-down and continued in proheterocysts and heterocysts. Vegetative cells

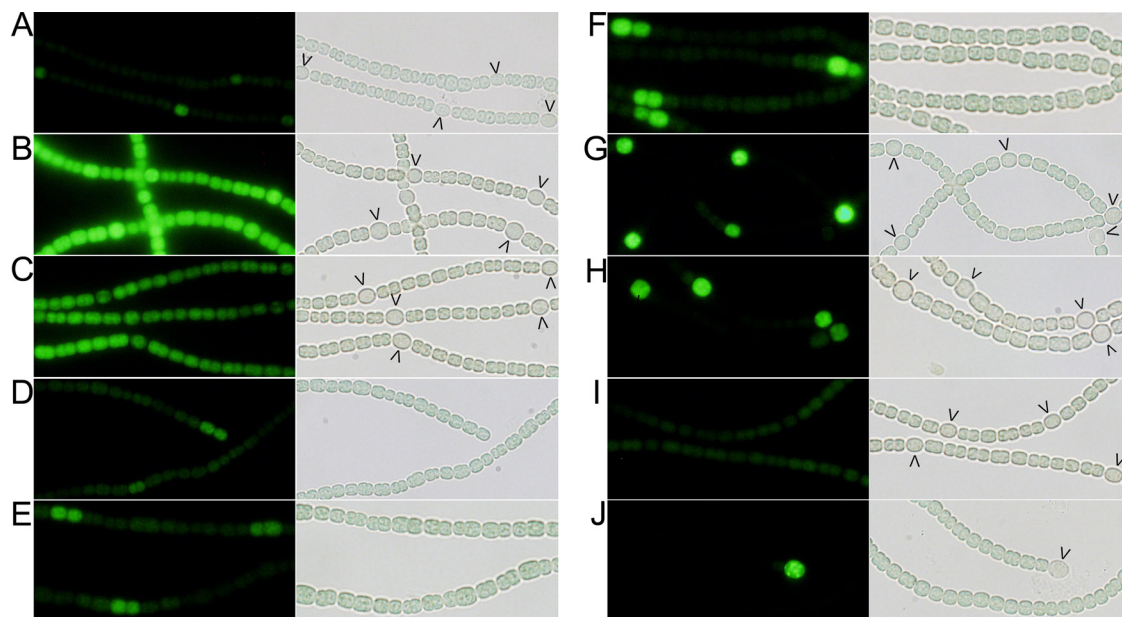


FIG. 3. Fluorescence micrographs (left images) and light transmission micrographs (right images) of *Anabaena* sp. PCC 7120 bearing (A) P_{hetR} -*gfp* in pSMC127, (B) the $-728/-696$ *tsp-gfp* fusion in pRR139, (C) the -184 *tsp-gfp* fusion in pRR151, (D to H) the -271 *tsp-gfp* fusion in pRR140, and (I) the $-728/-696(+132)$ *tsp-gfp* fusion in pRR149 and (J) the *patA* mutant UHM101 bearing the -271 *tsp-gfp* fusion in pRR140. The images in panels A, B, C, G, I, and J were obtained 18 h after combined nitrogen was removed from the culture. The images in panels D, E, F, and H were obtained 4, 8, 12, and 24 h, respectively, after combined nitrogen was removed from the culture. The arrowheads indicate proheterocysts and heterocysts.

between proheterocysts or heterocysts were dark except for the occasional cells midway between two heterocysts, which would presumably also differentiate in a subsequent round of heterocyst formation. The same progression of fluorescence from the -271 *tsp-gfp* fusion was observed even when ammonia was added to a concentration of 2 mM 14 h after nitrogen starvation began, just after the time of commitment to heterocyst differentiation (data not shown).

The *gfp* fusion with both the -271 and -184 *tsp*s showed fluorescence in vegetative cells, proheterocysts, and heterocysts, and the fluorescence was brighter in the proheterocysts and heterocysts (data not shown). These results indicate that transcription from *tsp* -271 alone is regulated in a cell-type-specific manner and suggest that patterned induction of *hetR* is primarily, if not solely, attributable to this autoregulated transcriptional start site. A control strain bearing a promoterless version of the fusion plasmid was dark under conditions similar to those used for the GFP fusions (data not shown).

A region in the *hetR* promoter downstream of *tsp*s -728 and -696 may have a negative regulatory role in transcription. As mentioned above, the level of GFP fluorescence observed with the $-728/-696$ *tsp* fusion was greater than the level of GFP fluorescence observed with the full-length promoter fusion of *hetR* to *gfp*. We speculated that the region of DNA downstream of the two *tsp*s may have a role in downregulating transcription. To verify this, the $-728/-696(+132)$ *tsp* fusion, which included an additional 132 bases of the *hetR* promoter downstream of the initial construct (Fig. 1), was introduced into the wild-type strain on a replicating plasmid, and GFP fluorescence was examined. As predicted, the GFP fluorescence from this fusion was greatly reduced under both nitro-

gen-replete and nitrogen starvation conditions compared to the GFP fluorescence from the original $-728/-696$ *tsp* fusion (Fig. 3I). A low level of fluorescence was observed in both vegetative cells and heterocysts. In an attempt to quantify the difference in transcription in the two *tsp* fusions, corresponding fusions to *lacZ* were constructed (Fig. 1) and introduced on a plasmid into the wild-type strain. However, there were fewer transconjugants containing the $-728/-696$ *tsp-lacZ* fusion, and they grew more slowly than other strains, including the wild-type strain containing the $-728/-696(+132)$ *tsp-lacZ* fusion. In addition, transconjugants exhibited dissimilar levels of β -galactosidase activity, which varied by more than 2 orders of magnitude or was absent in some cases. In contrast, transconjugants containing the *lacZ* fusion containing the additional downstream 132-bp region grew normally and exhibited similar levels of β -galactosidase activity that were roughly 20- to 60-fold lower than the levels exhibited by some of the strains carrying the $-728/-696$ *tsp-lacZ* fusion. These results suggest that transcription from the $-728/-696$ *tsp-lacZ* fusion negatively affects growth and that the presence of the extra 132-bp region limits transcriptional reporter activity.

PatA is required for transcription from *tsp* -271 in intercalary cells. In order to determine if the heterocyst regulator PatA plays a role in the regulation of transcription from one or more of the *tsp*s of *hetR*, transcription from the *hetR* *tsp*s was analyzed in a *patA* mutant. Transcription from all four *tsp*s was detected by RACE with RNA from both the wild type and the *patA* mutant, but not in the negative control, a strain lacking *hetR*. However, the intensity of the band corresponding to the -271 transcript was lighter in the *patA* mutant (Fig. 4A). Because RACE is an amplification procedure and therefore

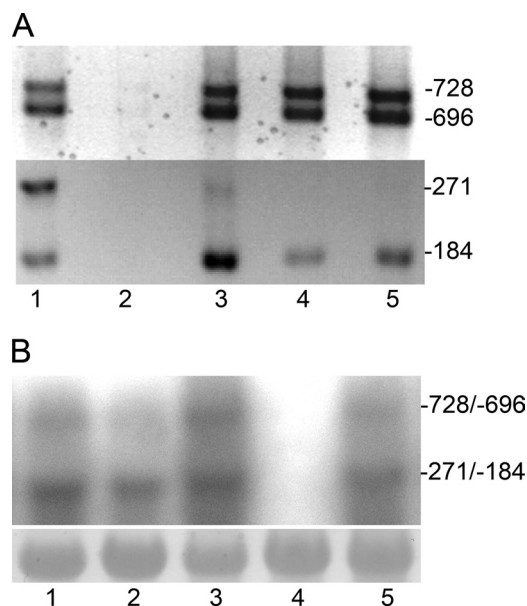


FIG. 4. Effects of PatA, PatS, and HetN on *hetR* transcription. (A) RACE results. Lane 1, wild-type strain; lane 2, *hetR* deletion strain UHM103; lane 3, *patA* deletion strain UHM101; lane 4, strain 7120PN, which overexpresses *hetN* from the *petE* promoter; lane 5, wild-type strain bearing pDR211, which overexpresses *patS* from the *petE* promoter. The upper panel shows products from the -728 and -696 tsps, and the lower panel shows products from the -271 and -184 tsps. (B) Northern blot results obtained using RNA isolated after 18 h of growth in BG11₀. Lane 1, strain 7120PN, which overexpresses *hetN* from the *petE* promoter; lane 2, wild type bearing plasmid pDR211, which overexpresses *patS* from the *petE* promoter; lane 3, wild type; lane 4, *hetR* deletion strain UHM103; lane 5, *patA* deletion strain UHM101. The lower panel shows the results for methylene blue-stained RNA as loading control.

could not be relied upon to report quantitative differences in transcription, other more quantitative approaches were used. Transcripts originating from the -271 and -184 tsps typically cannot be resolved on a Northern blot (4) (Fig. 4B). Therefore, the -271 *tsp-lacZ* fusion was introduced into the wild type and into the *patA* mutant on a replicating plasmid. Differences in β -galactosidase activity between the two strains clearly showed that the level of transcription from *tsp -271* was significantly lower in a *patA* mutant (Fig. 5A). There was substantial up-regulation of transcription from *tsp -271* in the wild type after nitrogen step-down up to about 15 h after induction of differentiation, after which the level of transcription started to level off (Fig. 5B). The *patA* mutant, however, showed only a modest increase in transcription from this *tsp*. A *patA* mutant produces primarily terminal heterocysts. The reduced level of transcription from the -271 *tsp* in the *patA* mutant correlates with the lower percentage of heterocysts in this strain. The lower levels of transcription could have been caused by insufficient induction of transcription from *tsp -271* in the intercalary cells of the filaments. To verify our hypothesis, the -271 *tsp-gfp* fusion was introduced on a replicating plasmid into the *patA* mutant, and the spatial transcription pattern was examined. GFP fluorescence was not detected under nitrogen-replete conditions. After induction of heterocyst differentiation, GFP expression was seen in the terminal cells of the filaments, which is where

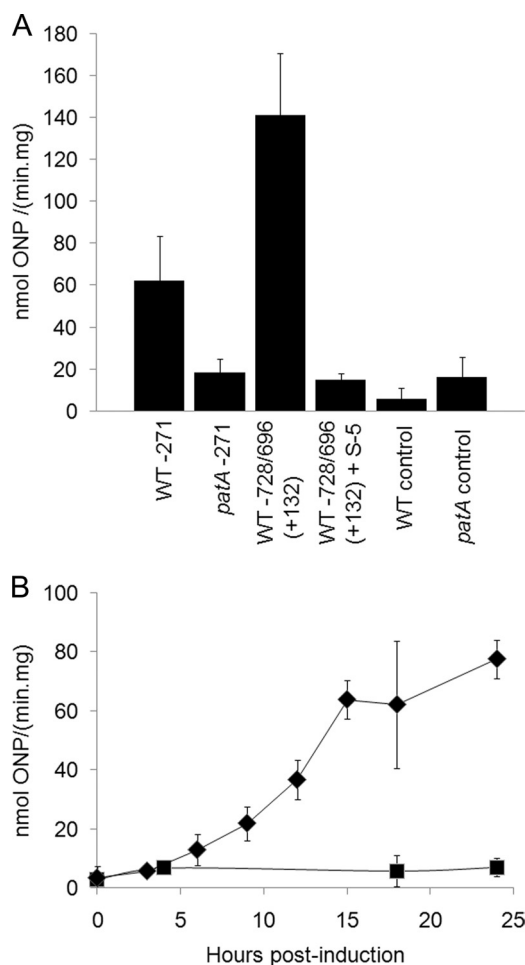


FIG. 5. (A) β -Galactosidase activity 18 h after induction of heterocyst differentiation for the wild type bearing the -271 *tsp-lacZ* fusion in plasmid pRR143, the *patA* mutant UHM101 bearing the -271 *tsp-lacZ* fusion in plasmid pRR143, the wild type bearing the $-728/-696(+132)$ *tsp-lacZ* fusion in plasmid pRR152, the wild type bearing the $-728/-696(+132)$ *tsp-lacZ* fusion in plasmid pRR152 with $6 \mu\text{M}$ PatS-5 included in the medium, the wild type bearing the control plasmid pNC101, and the *patA* mutant UHM101 bearing control plasmid pNC101. WT, wild type; S-5, PatS-5; ONP, *o*-nitrophenol. (B) Transcription from the -271 *tsp* at different times after induction of heterocyst differentiation. Diamonds, β -galactosidase activity of the wild type bearing the -271 *tsp-lacZ* fusion; squares, β -galactosidase activity of the wild type bearing control plasmid pNC101.

heterocyst differentiation primarily occurs in this strain (Fig. 3J). Other than occasional intercalary cells, which presumably would also differentiate into heterocysts, GFP fluorescence was not discernible in intercalary cells of the filaments. However, the *gfp* expression from the $-728/-696$ *tsp* fusion or the -184 *tsp* fusion was similar to that observed in the wild type (data not shown). This demonstrates that PatA is required for normal patterned transcription from *tsp -271* and underscores the tight nature of the control of transcription from this *tsp*.

PatS and HetN affect transcription from multiple tsps of *hetR*. The regulatory proteins PatS and HetN impact the spatiotemporal expression of HetR (6, 13, 16). To determine how each protein specifically impacts transcription from the four tsps of *hetR*, RACE was performed using RNA isolated 18 h

after induction from a strain overexpressing *hetN* from the chromosome (strain 7120PN) or from the wild-type strain bearing plasmid pDR211, which was used to overexpress *patS* from a copper-inducible promoter. The transcripts from *tsp* -184, -696, and -728 were detected in both strains (Fig. 4A). The band corresponding to transcripts from *tsp* -271 was absent in both 7120PN and the strain overexpressing *patS*, showing that PatS and HetN prevent detectable levels of transcription from *tsp* -271.

In a Northern blot containing the RNA obtained from these strains, the lower band, which corresponded to transcripts from *tsp* -271 and -184, was detected in both strains (Fig. 4B). Since the RACE results show that *tsp* -271 is turned off in these two strains, this band likely corresponded to transcripts originating from *tsp* -184. The upper band, which corresponded to transcripts from *tsp* -728 and -696, was similar to the band from the wild type for the strain overexpressing *hetN*. However, this band was lighter in the strain overexpressing *patS*.

In order to corroborate the Northern blot results, the wild-type strain bearing the -728/-696(+132) *tsp-lacZ* fusion on a replicating plasmid was grown in medium supplemented with 6 μ M PatS pentapeptide (PatS-5) (31), and β -galactosidase assays were performed. The β -galactosidase activity was significantly lower in the wild-type strain grown with PatS-5 added exogenously than in the same strain grown without PatS-5 (Fig. 5A). These results suggest that in addition to its negative impact on transcription from *tsp* -271, PatS downregulates transcription from one or both of the two distal *tsp*s as well.

The -271 *tsp* is required for normal numbers of heterocysts, the timing of patterning, and commitment to differentiation. To investigate whether transcription from the -271 *tsp* is involved in creation of the periodic pattern of heterocysts or instead is a consequence of an existing pattern, two strains that lacked regions of DNA around the -271 *tsp* were created. Plasmids containing the *hetR* coding and promoter regions lacking either nucleotides -295 to -268 or nucleotides -317 to -268 were constructed and recombined into the chromosomal *hetR* locus of a *hetR* deletion strain. The results obtained with the two single-recombinant strains were indistinguishable, so these strains are referred to below collectively as the -271 *tsp* deletion strains. RACE using RNA extracted 48 h after transfer to fixed-nitrogen-free media indicated that transcription from the -271 *tsp* did not occur in the -271 *tsp* deletion strains, whereas the transcription from the -184, -696, and -728 *tsp*s, as indicated by RACE, was similar to that of the wild type (data not shown). In both strains morphologically distinct heterocysts did not appear until 48 h after nitrogen starvation began (Fig. 6A and B), compared to 24 h for the wild type, and addition of ammonia to a final concentration of 2 mM 14 h after nitrogen starvation began prevented differentiation of heterocysts (data not shown). In contrast, the wild type forms cells committed to differentiation in a pattern prior to 14 h, after which addition of a source of fixed nitrogen does not prevent differentiation (30). The percentage of heterocysts formed by the -271 *tsp* deletion strains was about 2.5% at 48 h, compared to 9% for the wild type. In addition, induction of transcription from the -271 *tsp* in isolated cells was not observed from the -271 *tsp* deletion strains with the *PhetR-gfp* fusion on a plasmid 24 h after removal of combined nitrogen

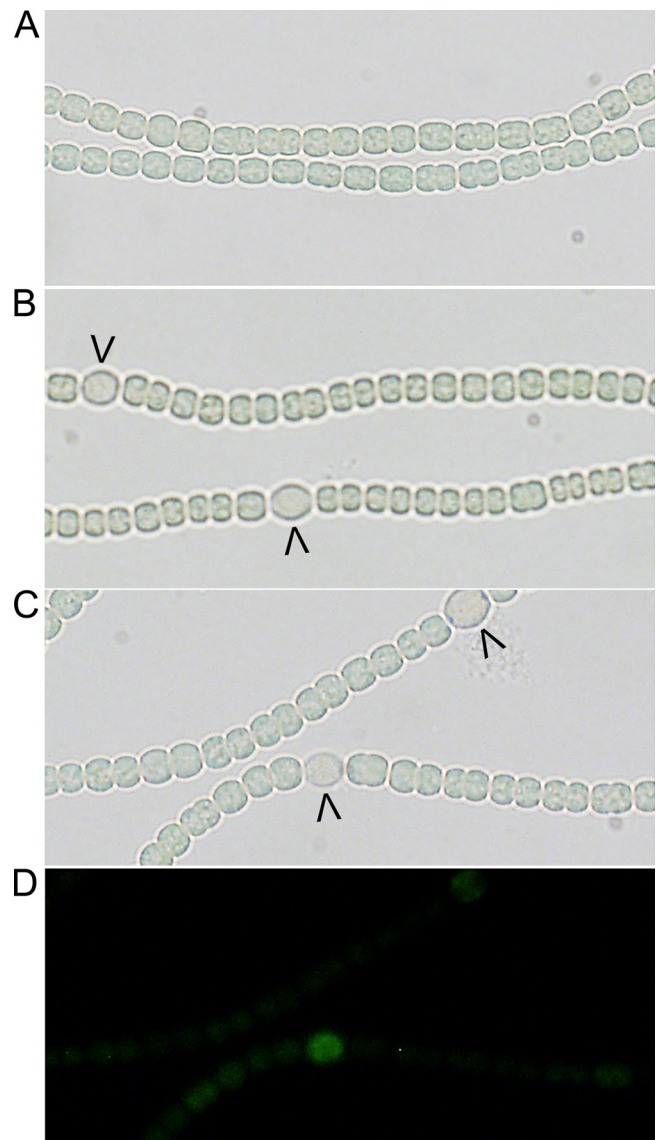


FIG. 6. Deletion of the -271 *tsp* region from the *hetR* promoter. (A and B) Photomicrographs of the -271 *tsp* deletion strain lacking nucleotides -295 to -268 of the *hetR* promoter region 24 h (A) and 48 h (B) after induction of heterocyst differentiation. (C and D) Light transmission micrograph (C) and fluorescence micrograph (D) obtained 48 h after combined nitrogen was removed from the -271 *tsp* deletion strain lacking nucleotides -295 to -268 of the *hetR* promoter region bearing P_{hetR} -*gfp* in a plasmid (pSMC127). The arrowheads indicate proheterocysts and heterocysts.

(data not shown). An increase in GFP fluorescence was observed in some cells 36 h after combined nitrogen was removed (Fig. 6C and D). Patterned fluorescence in the wild type with the same construct was visible 8 h after combined nitrogen was removed. Subsequently, the corresponding double-recombinant strains UHM156 and UHM157, which differ from the wild type by having nucleotides -295 to -268 and nucleotides -317 to -268, respectively, of the *hetR* promoter deleted cleanly from the chromosome, were isolated and shown to have phenotypes indistinguishable from those of the single-recombinant strains used in the experiment described above. These results indicate that the -271

tsp is required for pattern formation, commitment to differentiation, and differentiation of a number of heterocysts similar to that seen in the wild type.

DISCUSSION

The spatiotemporal regulation of transcription from the four transcriptional start sites of *hetR* was investigated to determine how each *tsp* affects cellular differentiation and pattern formation in *Anabaena* sp. PCC 7120. Induction of *hetR* expression through use of inducible promoters, such as P_{petE} (5) and P_{nir} (23), on a multicopy plasmid triggers heterocyst differentiation even under conditions under which differentiation does not normally take place. Under such conditions, however, the frequency and pattern of heterocysts are atypical. Differentiation of heterocysts in a wild-type pattern requires first that expression of HetR is localized to select cells and then that the levels of expression are sufficient to irreversibly commit cells to differentiate into heterocysts. Differential expression of *hetR* in response to the timing of nitrogen starvation and the relative position of a cell in a filament is facilitated by multiple transcription start points.

When fixed nitrogen in a form that can be readily incorporated into carbon skeletons, such as ammonia, is present at a concentration sufficient for robust growth, expression of *hetR* is primarily from the -184 *tsp* (5, 19). Presumably, a low, basal level of *hetR* expression maintains the cells of the filament in a state primed to respond quickly to nitrogen starvation. It is not likely that transcription from the other *tsp*s of *hetR* has a significant role in maintaining basal *hetR* expression levels. Transcription is not detected from the -271 *tsp* under nitrogen-replete conditions. The RACE experiments demonstrated that transcription occurs from the -728 and -696 *tsp*s even under nitrogen-replete conditions. However, the level of transcription is quite low, as demonstrated by other workers using Northern blots and primer extension (5, 19) and as demonstrated in this study with reporter fusions. The dramatic differences in transcription observed in nitrogen-replete conditions with the $-728/-696$ and $-728/-696(+132)$ *tsp*-reporter fusions indicate that a region of DNA downstream of these *tsp*s is necessary for limiting transcription from the *tsp*s under nitrogen-replete conditions.

The two distal *tsp*s of *hetR* appear to form the nitrogen-status-sensing and response arm of the *hetR* transcription apparatus. The signal for nitrogen starvation is transmitted to *hetR* by NtcA via NrrA, resulting in induction of transcription from the *tsp*s located at nucleotides -728 and -696 (7, 8). The data for *tsp-gfp* fusions suggest that transcription from these two *tsp*s occurs at roughly the same level along the filament and is not spatially regulated, although when the data obtained for these fusions are interpreted, it should be borne in mind that each of these *tsp* fusions lacks parts of the promoter that may contribute to its regulation. On the other hand, our results demonstrated that the -271 *tsp* is differentially regulated based on a cell's position in a filament. Soon after induction, clusters of fluorescent cells are present in filaments carrying the -271 *tsp-gfp* fusion, demonstrating that transcription from the -271 *tsp* is upregulated in several groups of contiguous cells prior to resolution of these clusters to produce single cells. This pattern of transcription is similar to that exhibited by *patS*

(30). Between 12 and 18 h after the onset of nitrogen starvation, transcription from this start site is localized to developing heterocysts. This time roughly coincides with the commitment of cells to differentiation. Subsequently, transcription from the -271 *tsp* is seen only in vegetative cells that are midway between heterocysts, and these cells presumably differentiate in a secondary round of differentiation. These results suggest that the patterned induction of *hetR* observed upon nitrogen deprivation is attributable primarily to this transcriptional start site. Intriguingly, when nitrate, which must be reduced before it can be incorporated into an organic molecule, is used as the fixed-nitrogen source rather than ammonia, filaments are maintained in a state in which clusters of cells express *hetR* from the -271 *tsp* (data not shown), demonstrating that the spatial induction of transcription from the -271 *tsp* can be maintained in an intermediate state. Our results are consistent with constitutive expression from the -184 *tsp*, which supplies a basal level of *hetR* mRNA under all growth conditions.

The network of genes involved in regulating differentiation and pattern formation has the potential to act as a genetic switch that converts a temporary input signal, such as nitrogen starvation, into a stable "on" or "off" gene expression response, leading to permanent alteration of cells (10). In genetic switches positive and/or negative feedback loops contribute to memory by the system. HetR forms positive and negative feedback loops with itself and with other regulatory proteins. In strains in which the -271 *tsp* was deleted from the chromosome, heterocyst differentiation was delayed along with patterned transcription from a $P_{hetR-gfp}$ reporter fusion. The percentage of heterocysts was also lower in these strains. Taken together, these results indicate that the -271 *tsp* is necessary for normal timing of pattern formation and morphological differentiation.

Heterocyst differentiation is an endpoint process leading to permanent genetic, morphological, and physiological changes in the differentiating cell. The heterocyst does not divide and cannot revert to being a vegetative cell. Between 9 and 14 h after induction of differentiation, cells become committed to differentiate, even if the initial starvation signal is removed (30). By using mathematical modeling, Brandman et al. (3) predicted that many biological systems use multiple interlinked loops because they generate reliable all-or-none switches that are both rapidly induced and more resistant to noise in the upstream signaling pathway than single positive feedback loops. When one of two loops regulating a pathway is slow and the other is fast, the fast loop is critical to the speed of transition from one state to the other, while the slow loop is essential for the stability of the "on" state (3). We suggest that the NtcA-mediated activation of transcription from the -728 and -696 *tsp*s generates a quick response to nitrogen starvation, while induction of transcription from the -271 *tsp* takes place at a lower rate and is necessary for the normal timing of irreversible commitment to differentiation.

Our results show that PatA, HetN, and PatS all regulate transcription from the -271 *tsp*, which in turn should affect the direct positive feedback loop discussed above. HetN and PatS have a negative effect on transcription from the -271 *tsp*, and PatA has a positive effect. These findings are consistent with the suggestion that PatA attenuates the inhibitory activities of PatS and HetN. It has been proposed that PatA mediates the

protein-protein interaction between HetR and HetF (22). It has also been proposed that HetF, a predicted protease, modifies HetR into a form that is less sensitive to inhibition by PatS and HetN. In the absence of *hetF*, transcription from the -271 tsp is not detected, and overexpression of *hetF* in a *patA* mutant results in bypass of the terminal heterocyst phenotype (22). Thus, it seems likely that PatA works through HetF to effect transcription from $tsp -271$. It has been proposed that PatS acts as a diffusible inhibitor of differentiation and interacts with HetR in pattern formation. Upregulation of *patS* transcription is dependent on *hetR* (13). It is likely that competition between HetR and PatS plays a significant role in localization of transcription from $tsp -271$. Although HetN does not play a major role in pattern formation, it appears to stabilize the pattern and regulate the sites of new heterocysts between existing heterocysts to maintain the pattern as a filament lengthens by preventing transcription from $tsp -271$. Transcription from the start sites of *hetR* is differentially regulated based on fixed-nitrogen levels in the medium and the relative position of a cell in a filament. Several genes involved in differentiation, such as *ntcA* (19, 21), *hetR* (5, 19), *glnA* (24), *patS* (30), and possibly the *hetZ-patU* cluster (31), are transcribed from multiple transcription start points. The use of multiple *tsp*s rather than differential regulation of a single *tsp* appears to be a common theme for the regulation of genes involved in the differentiation and patterning of heterocysts.

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