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NtcA-Regulated Heterocyst Differentiation Genes *hetC* and *devB* from *Anabaena* sp. Strain PCC 7120 Exhibit a Similar Tandem Promoter Arrangement[▽]

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Transcription of the *hetC* gene, whose product is required for heterocyst differentiation, takes place from a long promoter region that includes the previously described HetR-independent, NtcA-activated promoter producing transcripts with a 5′ end corresponding to position −571 with respect to the translational start site of *hetC*. Northern blot analysis indicated that the accumulation of *hetC* transcripts depends on HetR, and a second transcriptional start site located at position −293 that leads to NtcA-dependent, HetR-dependent inducible transcription of *hetC* was identified. Upon nitrogen stepdown, expression of a P_{hetC}::*gfp* fusion was transiently induced in specific cells that were differentiating into heterocysts, both when the whole promoter region (containing transcription start points −571 and −293) or a short version (containing only the transcription start point −293) was used. Expression of *hetC* from the −293 position was delayed in a strain bearing a deleted promoter region lacking sequences upstream from position −570. Such a strain was still able to differentiate functional heterocysts and to grow diazotrophically, although diazotrophic growth was impaired under certain conditions. Similarly, a second, NtcA-dependent, HetR-dependent transcriptional start site was identified at position −454 in the promoter region upstream from the *devBCA* operon encoding an ABC transport system involved in heterocyst maturation, in which an NtcA-dependent promoter producing transcripts starting at position −704 had been previously noted. Thus, the *hetC* and *devBCA* promoter regions exhibit similar tandem promoter arrangements.

Nitrogen fixation in many cyanobacterial strains, including *Anabaena* spp., requires differentiation of a specific cell type, called a heterocyst, in which the machinery for nitrogen fixation is confined. Heterocyst differentiation is integrated in a suite of responses to nitrogen deficiency that take place when ammonium, the preferred nitrogen source, is not available. Assimilation of different nitrogen sources is globally regulated in cyanobacteria by NtcA, a transcriptional regulator of the cyclic AMP receptor protein (CAP) family that, in the absence of ammonium, activates the expression of genes required for the assimilation of alternative nitrogen sources, including those required for heterocyst differentiation. NtcA binds upstream from the activated transcriptional start sites, and a consensus sequence for NtcA binding has been defined (for reviews, see references 18 and 19). In many cases, NtcA-activated promoters are similar to class II CAP-dependent promoters, in which the transcription factor binds to a sequence centered about 41 nucleotides upstream from the transcription start point (TSP) to interact with RNA polymerase (8). The differentiation of heterocysts begins shortly after nitrogen deprivation and, under our experimental conditions, requires about 24 h to complete. The formation of functional mature heterocysts requires the sequential activation of a large number of genes involved in regulatory, structural, or enzymatic aspects of heterocyst differentiation and function (1, 35, 37).

Early events in heterocyst differentiation in *Anabaena* sp. strain PCC 7120 require increased expression of *hetR* (4, 5), encoding a positively acting factor that exhibits protease (29, 38) and DNA-binding (20) activities in vitro, and of *ntcA* (17, 34). Inductions of the two genes are mutually dependent in the context of heterocyst differentiation (23) and take place mostly in cells that are differentiating into heterocysts (4, 25). Because the promoter of *hetR* does not exhibit the features characteristic of NtcA-activated promoters, the molecular basis for its dependence on NtcA is currently unknown. However, the involvement of the NrrA protein, which is the product of the NtcA-dependent *nrrA* gene, in *hetR* expression could, at least in part, explain the requirement for NtcA (12, 13, 22).

Mutation of the *hetC* gene, encoding a protein similar to bacterial ABC exporters, blocks heterocyst differentiation under nitrogen deficiency (21). Induction of *hetC* takes place upon nitrogen stepdown and depends on NtcA (24). A consensus class II NtcA-activated promoter that produces transcripts with a 5′ end located in position −571 with respect to the *hetC* reading frame that can be readily detected, under our experimental conditions, after 3 h of nitrogen stepdown has been described for *hetC* (24). Transcription from this NtcA-activated promoter does not require HetR in vivo (23). However, some previous observations by Khudyakov and Wolk (21) using a *luxAB* fusion close to the 3′ end of the *hetC* gene (a ScaI site) (Fig. 1) indicated that, upon nitrogen stepdown, there was a much lower rate of increase of luminescence in a

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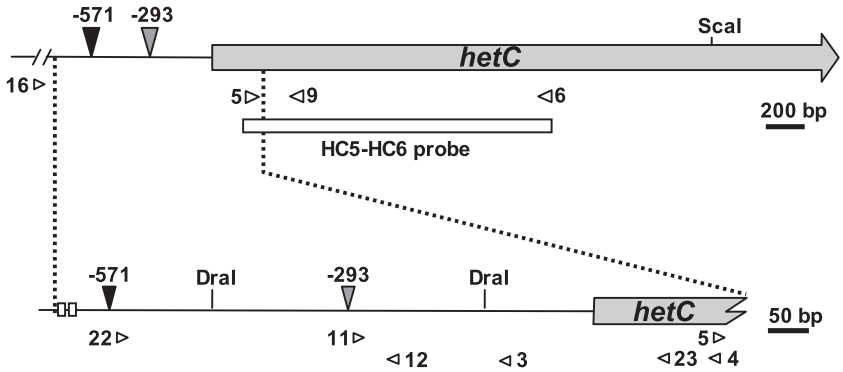

FIG. 1. Scheme of the *hetC* region of *Anabaena* sp. strain PCC 7120. The *hetC* gene is shown in gray. The positions of TSPs −571 (black triangle) and −293 (gray triangle; see below) are indicated, as well as the NtcA binding site located upstream from position −571 (small white boxes). The positions of some relevant oligonucleotides used in this work (HC3 to HC23) are indicated as numbered arrowheads pointing right or left according to the direction of extension. The probe used in the Northern blot analysis shown in Fig. 2A (HC5-HC6) is also indicated.

hetR than in a wild-type background, indicating a certain dependence on HetR (21). Additionally, it has been shown that expression of *hetC*::*gfp* fusions takes place most strongly in proheterocysts and heterocysts (33, 36).

The *devBCA* cluster encodes an ABC-type transporter involved in the deposition of the glycolipid layer required for maturation of a functional heterocyst (15). A consensus class II NtcA-dependent promoter producing transcripts that start at position −704 has been described upstream of *devB*, and accumulation of *devBCA* transcripts depends on HetR (9, 16). Thus, the promoter regions upstream of *hetC* and *devB* contain consensus NtcA-activated promoters, HetR independent in the case of *hetC*, which has been investigated in vivo (23), that could produce transcripts with very long, presumably untranslated leaders. Also, for both genes, there is evidence pointing to accumulation of the corresponding transcripts being influenced by HetR.

We further investigated the transcription of *hetC* and showed, by means of Northern blot hybridization, that the accumulation of *hetC* transcripts during heterocyst differentiation depends on the HetR protein. Also, a new NtcA-dependent promoter that produces transcripts starting at position −293 with respect to the *hetC* reading frame, whose abundance depends on HetR, has been identified. The timing and regulation of expression observed from such a transcriptional start site is consistent with Northern blot data. We prepared constructs bearing deletions of the region upstream from the transcriptional start site located at −571 and found that transcription from position −293 can take place in the absence of such an upstream region. We also determined that, upon nitrogen stepdown, induction of the expression of *hetC* from this previously unidentified promoter takes place in differentiating heterocysts. Finally, we determined that the NtcA-regulated *devBCA* operon exhibits a setting for transcription initiation similar to that of the *hetC* gene, with the previously described promoter that produces transcripts starting at position −704 being independent of HetR and a newly identified NtcA-dependent, HetR-dependent promoter producing transcripts from position −454.

MATERIALS AND METHODS

Strains and growth conditions. This study was carried out with the heterocyst-forming cyanobacterium *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 and three mutant derivatives unable to differentiate heterocysts, strain CSE2 (an insertional mutant of the *ntcA* gene) (17), strain 216 (which bears a point mutation in the *hetR* gene) (5), and strain DR884a (an insertional mutant of the *hetR* gene) (4). They were grown photoautotrophically at 30°C in $BG11_0C$ medium (BG11 medium [28] without $NaNO_3$ and supplemented with 0.84 g of $NaHCO_3$ $liter^{-1}$) supplemented with 6 mM NH_4Cl plus 12 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH buffer (pH 7.5), bubbled with a mixture of CO_2 and air (1% [vol/vol]), and supplemented with 2 μg of streptomycin ml^{-1} and 2 μg of spectinomycin ml^{-1} in the case of strain CSE2 or 5 μg of neomycin ml^{-1} in the case of strain DR884a. Exconjugants were selected and maintained in solid BG11 medium (solidified with 1% Difco agar) in the presence of 5 μg of streptomycin ml^{-1} and 5 μg of spectinomycin ml^{-1} or 25 μg of neomycin ml^{-1}.

For RNA isolation, cells growing exponentially (about 3 to 5 μg of chlorophyll *a* ml^{-1}) in $BG11_0C$ medium supplemented with NH_4Cl were harvested at room temperature and either used directly (time zero) or washed with $BG11_0C$ medium, resuspended in $BG11_0C$ (nitrogen-free) medium, and further incubated under culture conditions for the number of hours indicated in each experiment.

Plasmids. A fragment from the *hetC* region comprising positions −980 to +90 with respect to the *hetC* translational start site was amplified by PCR using oligonucleotides HC16 (which introduces a ClaI site upstream from position −980) and HC23 (which introduces an EcoRV site in positions +82 to +87) and chromosomal DNA as a template and cloned into the pMBL-T vector (Dominion-MBL), producing pCSAM143. (All oligonucleotide primers are listed in Table 1.) Two mutant versions of the same fragment, with either the sequence of the NtcA binding site or the −10 box of the promoter located upstream from the TSP at position −571 altered, were amplified as described previously (2) using the mutagenic oligonucleotides HC18 and HC19 or the mutagenic oligonucleotides HC20 and HC21, respectively. The flanking oligonucleotides were HC16 and HC23 (see above) in both cases. The corresponding fragments were cloned into the pMBL-T vector, producing pCSAM144 and pCSAM145, respectively. A short version of the promoter was amplified by PCR using oligonucleotides HC22 (which introduces a ClaI site just upstream from position −570) and HC23 (see above) and cloned into the pMBL-T vector, producing pCSAM146. To ensure that only the desired mutations had been introduced, the four versions of the promoter were completely sequenced.

In order to obtain translational fusions of the first portion of the *hetC* gene (29 amino acid residues) and the *gfp*-mut2 gene (11), ClaI-EcoRV fragments from pCSAM143, pCSAM144, pCSAM145, and pCSAM146, comprising wild-type and modified versions of the *hetC* promoter, were cloned into the ClaI/EcoRV-digested plasmid pCSEL21 (25), producing plasmids pCSAM147, pCSAM148, pCSAM149, and pCSAM150, respectively. EcoRI fragments from pCSAM147, pCSAM148, pCSAM149, and pCSAM150 containing the *hetC* promoter plus the *hetC*::*gfp* translational fusion were cloned into the EcoRI-digested

TABLE 1. Deoxyoligonucleotide primers used in this work

Primer name	Sequence[a]	Position with respect to the corresponding ATG
HC3	5′-CGGCATTTTAATGTACTGCC-3′	−85 to −104 (*hetC*)
HC4	5′-GCCGAACTACCCAGTTTTGG-3′	+160 to +141 (*hetC*)
HC5	5′-AGAGTTGAGCCAAAACTGG-3′	+132 to +150 (*hetC*)
HC6	5′-GTAAGGGTAACTGCAACG-3′	+1729 to +1712 (*hetC*)
HC9	5′-TCAGCAGTTGTCGTAGAGATGAC-3′	+416 to +394 (*hetC*)
HC11	5′-GGTGATTCAACAAAATATAGATAG-3′	−290 to −266 (*hetC*)
HC12	5′-CCTGTTGATTATTCATGAG-3′	−207 to −225 (*hetC*)
HC16	5′-ATCGATACCTATCTCCGCCCTATG-3′	−980 to −963 (*hetC*)
HC18	5′-AATCT**CA**TACATGAGATACACAATAGC-3′	−624 to −598 (*hetC*)
HC19	5′-CTCATGT**ATG**AGATTTTTTCCGATAGC-3′	−610 to −636 (*hetC*)
HC20	5′-GCTT**GG**GTA**G**CTCTCTCTTGG-3′	−587 to −567 (*hetC*)
HC21	5′-GAGAG**C**TAC**C**CAAGCAAATATAAATG-3′	−573 to −598 (*hetC*)
HC22	5′-ATCGATTTGGGTGGGATTCTG-3′	−570 to −555 (*hetC*)
HC23	5′-GAGGATATCCACTTCTGGAG-3′	+90 to +71 (*hetC*)
DB1	5′-CCCCCTACTCCCTTTCC-3′	−825 to −809 (*devB*)
DB13	5′-TCCGTCACCCTTGACATGG-3′	+17 to −2 (*devB*)
DB15	5′-CCACAATGTACTCGTTTCTG-3′	−209 to −228 (*devB*)
OdevB7120	5′-GAAGAGGTTCTATCAAAGG-3′	−519 to −537 (*devB*)
GFP4	5′-CAAGAATTGGGACAACTCC-3′	+46 to +28 (*gfp*)
RACE DNA primer (B)	5′-GGTATTGCGGTACCCTTGT-3′	
RACE RNA adaptor	5′-CUAGUACUCCGGUAUUGCGGUACCCUUGUA CGCCUGUUUUAUA-3′	

[a] Nucleotide changes are indicated in boldface type. Introduced restriction sites are underlined.

replicative vector pDUCA7 (7), producing pCSAM161, pCSAM162, pCSAM163, and pCSAM164, respectively.

Plasmid pCSAM155, used to determine the 5′ ends of *devB* transcripts in primer extension assays, contains an 842-bp fragment corresponding to positions −825 to +17 with respect to the translational start site of the *devB* gene from *Anabaena* sp. strain PCC 7120. This fragment was amplified by PCR using oligonucleotides DB1 and DB13 (Table 1) and chromosomal DNA as a template and cloned into plasmid pMBL-T (Dominion-MBL).

DNA and RNA isolation and manipulation. Total DNA (10) and RNA (23) from *Anabaena* sp. strain PCC 7120 and its derivatives was isolated as previously described. Plasmid isolation from *Escherichia coli*, transformation of *E. coli*, digestion of DNA with restriction endonucleases, ligation with T_4 ligase, sequencing of plasmid DNA, and PCR amplification were performed by standard procedures (2).

Northern blotting and hybridization. Northern analysis was carried out after the separation of RNAs in agarose gels according to standard procedures (2). The *hetC* probe was a PCR product amplified using oligonucleotides HC5 and HC6 and pCSAM86 (24) as template. The fragment was ^{32}P labeled with a Ready-to-Go DNA-labeling kit (Amersham Biosciences). Images of radioactive filters were obtained and quantified using a Cyclone storage phosphor system and OptiQuant image analysis software (Packard).

Primer extension analysis. Primer extension analysis of *hetC* and *devB* transcripts was carried out as described previously (24). The oligonucleotides used as primers for the *hetC* transcripts were HC3, HC4, and HC9. The oligonucleotides used as primers for the *devBCA* transcripts were OdevB7120, DB13, and DB15. Oligonucleotide GFP4 was used when analyzing the expression of fusions to the *gfp* gene.

5′ rapid amplification of cDNA ends (RACE). Reverse transcription-PCR amplification of transcripts ligated to short 5′ RNA adaptors was carried out as described previously (3). Two reactions were set up for each RNA sample. One of them included treatment with tobacco acid pyrophosphatase (TAP); 20 μg of total RNA was treated with 20 units TAP (Epicentre) for 2 h at 37°C in a volume of 50 μl. The reaction mixtures were extracted with phenol-chloroform, and RNA was precipitated with ethanol and sodium acetate and dissolved in 10 μl water. Sixty picomoles of 5′ RNA adaptor (Table 1) (Sigma Proligo) was added to the RNA sample, and ligation was performed in the presence of 80 units of T_4 RNA ligase (Amersham) for 16 h at 15°C. The reaction mixture was extracted once with phenol-chloroform and once with chloroform, and RNA was precipitated with ethanol and sodium acetate and dissolved in water. Retrotranscription reactions were carried out with 6.5 μg of RNA ligated to the adaptor and several gene-specific primers (HC3 or HC4) using Superscript II reverse transcriptase (Invitrogen). Five-microliter portions (about 1 μg RNA) of the reverse transcription reaction mixtures were used as templates for subsequent PCRs with a gene-specific primer and a primer corresponding to sequences from the RNA adaptor (Table 1). PCR products of the expected sizes were isolated from agarose gels and cloned into the pGEM-T vector (Promega) for sequencing. The first nucleotide following the adaptor sequence was taken as the TSP.

Construction of strains bearing P_{hetC}::*gfp* fusions. The replicative plasmids pCSAM161 (with a wild-type promoter region), pCSAM162 (with a mutated NtcA box), pCSAM163 (with a mutated −10 box), and pCSAM164 (a short version of the promoter region), bearing different P_{hetC}::*gfp* fusions, were transferred to *Anabaena* by conjugation (14), and the exconjugants were selected in BG11 medium supplemented with neomycin. In order to corroborate that replicative plasmids had not integrated into the chromosome through homologous recombination, PCRs with different combinations of oligonucleotides corresponding to pDUCA7 and chromosomal sequences were carried out using DNA isolated from cells used in the experiment (see Fig. 3 and data not shown).

Construction of strains bearing duplications of the *hetC* promoter region. EcoRI fragments from pCSAM147 (with a complete wild-type *hetC* promoter region) or pCSAM150 (with a short version of the *hetC* promoter), containing the corresponding versions of the *hetC* promoter plus the *hetC*::*gfp* translational fusion, were cloned into the EcoRI-digested, CS.3 (Sm^r Sp^r gene cassette)-containing pCSV3 suicide plasmid (for a description, see reference 25), producing pCSAM157 and pCSAM160, respectively (see Fig. 4). Both plasmids were transferred to *Anabaena* sp. strain PCC 7120 by conjugation. Exconjugants were selected in BG11 medium supplemented with streptomycin and spectinomycin, and their genomic structures were confirmed by PCR. The resulting strains were named CSAM157 and CSAM160, respectively.

Fluorescence determinations. The accumulation of green fluorescent protein (GFP) reporter was analyzed by laser confocal microscopy as previously described (22). GFP was excited using the 488-nm line supplied by an argon ion laser, and the fluorescent emission was monitored by collection across windows of 500 to 540 nm (GFP imaging) and 630 to 700 nm (cyanobacterial autofluorescence).

Alcian blue staining. Alcian blue was used to stain heterocyst-specific envelope polysaccharides. A 0.5% Alcian blue (Sigma) solution in 50% ethanol was mixed with an equal volume of cell suspension before examination under the microscope.

RESULTS

Effects of HetR on expression of *hetC*. The expression of *hetC* in *Anabaena* sp. strain PCC 7120 and the *hetR* strain 216 was analyzed by Northern blotting using as a probe an internal

fragment of the *hetC* coding sequence (probe HC5-HC6) (Fig. 1). In the wild-type strain, as previously described (21, 24, 36), expression of *hetC* was very low in the presence of ammonium and was transiently induced upon nitrogen deprivation, reaching the highest level after ca. 9 h of nitrogen stepdown (Fig. 2A). In the *hetR* strain, a lower level of induction was observed, and the transcript remained at a low level for up to 24 h after nitrogen stepdown. Although according to previous data HetR was not required for induction of transcription initiation from the −571 TSP (23), the results shown in Fig. 2A indicate that HetR influences the accumulation of transcripts corresponding to the coding sequence of *hetC*. Such accumulation peaked relatively late with respect to transcription from position −571, which takes place to a similar extent from 3 h up to 24 h after nitrogen stepdown (23, 24).

Identification of a second transcription start site for *hetC*. The Northern blot results shown in Fig. 2A suggested that a previously undetected promoter could contribute to the production of transcripts covering the coding region of *hetC*. Primer extension assays were performed using oligonucleotide HC4 and RNA samples from the wild-type, *ntcA*, and *hetR* strains. Figure 2B shows that previously undetected inducible transcripts with a 5′ end located at position −293 with respect to the translational start site of *hetC* could be identified in the wild-type strain, but not in the *ntcA* or *hetR* mutant. Such transcripts were absent in the presence of ammonium (not shown) and were detected after 3 h of nitrogen deficiency and at higher levels after 9 h of nitrogen deficiency. The same 5′ end was identified in primer extension assays using oligonucleotides HC3 and HC9 (not shown). The dependence on HetR observed for transcription from position −293 was consistent with the regulation observed for *hetC* transcripts in the Northern blot experiment shown in Fig. 2A.

Transcripts with 5′ ends corresponding to position −293 were further analyzed in order to determine whether those species corresponded to a true transcriptional start site or whether they could be the result of processing of longer transcripts originating at position −571. A procedure based on 5′ RACE of RNA samples ligated to short 5′ RNA adaptors was used (3). In this procedure, RNA samples were subjected to treatment with TAP (which cleaves pyrophosphate bonds from 5′triphosphate RNA molecules), ligated to 5′ RNA adaptors, and retrotranscribed with gene-specific oligonucleotides as primers. Subsequent PCR using a gene-specific primer and a primer corresponding to sequences from the RNA adaptor differentiated 5′ ends to which the 5′ RNA adaptor could be ligated in both TAP-treated and untreated samples (processed RNAs) from 5′ ends to which the 5′ RNA adaptor could be ligated only in TAP-treated samples (true transcriptional start sites) (see Materials and Methods for details). The results obtained with RNA samples isolated from the wild-type and *hetR* strains after 9 h of nitrogen deficiency are shown in Fig. 2C. Reverse transcription was carried out with oligonucleotide HC3 or HC4 (Fig. 1). The results shown indicate that a PCR product of the expected size was observed after subsequent PCR using oligonucleotides HC4 (or HC12 [not shown]) and B (corresponding to the sequence of the RNA linker) when TAP-treated RNA was used but not when untreated RNA was used as a template. As a control, PCR products amplified with oligonucleotides HC3 and HC11 (Fig. 1 shows their positions)

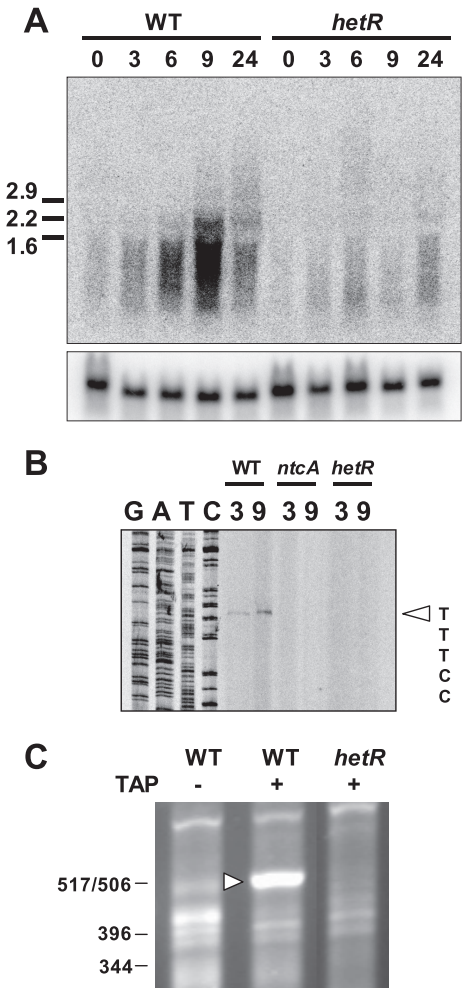

FIG. 2. Analysis of expression of the *hetC* region in *Anabaena* sp. strain PCC 7120 and mutant strains CSE2 (*ntcA*), 216 (*hetR*), and DR884a (*hetR*). RNA was isolated from ammonium-grown cells (lanes 0) or from ammonium-grown cells incubated in combined nitrogen-free medium for 3, 6, 9, or 24 h. (A) Northern blot analysis in strains PCC 7120 and 216. The *hetC* probe was a PCR fragment corresponding to fragment HC5-HC6. The samples contained 50 μg of RNA. Hybridization to *rnpB* (32) served as a loading and transfer control (lower blot). The sizes of ribosomal RNAs (in kilobases) are indicated on the left. (B) Primer extension analysis of the *hetC* upstream region in wild-type strain PCC 7120 and mutant strains CSE2 and DR884a. Oligonucleotide HC4 (the position is shown in Fig. 1) was used as a primer in assays containing 25 μg of RNA. A sequencing ladder was generated with the same oligonucleotide and plasmid pCSAM83 (24). The triangle points to the 5′ end identified at position −293 with respect to the *hetC* translational start site. The sequence of the 5′ end is included. (C) Analysis of the *hetC* gene transcriptional start site at position −293 by 5′ RACE. RNA isolated from ammonium-grown cells of the wild type and the *hetR* strain 216 incubated in the absence of combined nitrogen for 9 h was treated (lanes +) or not (lanes −) with TAP and ligated to an RNA adaptor with a known sequence. Samples were then used as templates for reverse transcription using the *hetC*-specific oligonucleotide HC4. The resulting cDNA was amplified by PCR using oligonucleotides HC4 and B (corresponding to the RNA adaptor ligated to RNA samples). The triangle points to PCR products present in wild-type TAP-treated samples but not in wild-type untreated samples or in samples from the *hetR* strain. Size standards in base pairs are indicated on the left. WT, wild-type strain PCC 7120.

were obtained in both cases (not shown). Southern blotting using a DraI-HC4 probe corresponding to positions −116 to +160 with respect to the translational start site of *hetC* confirmed that the identified PCR products corresponded to the *hetC* region (not shown). In order to confirm the 5′ end of the RNA molecules under analysis, PCR products obtained with oligonucleotides HC3 and B or HC12 and B were cloned in the pGEM-T vector, and several clones were sequenced. The sequences obtained corresponded to those of the 5′ RNA adaptor ligated to position −293 with respect to the translational start site of *hetC*, thus confirming that there was, in fact, a true transcriptional start site at position −293. Furthermore, Fig. 2C shows that when RNA isolated from the *hetR* strain after 9 h of nitrogen deficiency was used in these experiments, the PCR products corresponding to transcripts originating around position −293 were not obtained. This observation is consistent with primer extension data (Fig. 2B) showing that there was no detectable transcription from position −293 in a *hetR* strain.

Construction and analysis of strains bearing altered versions of the *hetC* promoter region fused to the *gfp* gene. It has been shown that induction of the expression of *hetC* upon nitrogen stepdown is mostly localized to proheterocysts (33, 36). In order to assess the roles of the two promoters identified for the *hetC* gene in the expression in specific cells, replicative plasmids bearing wild-type and mutated versions of the *hetC* promoter region in which the *hetC* gene was translationally fused to the *gfp* gene were prepared. Modified versions of the promoter included mutations in the NtcA box or the −10 box upstream from position −571 or a deletion of the region upstream from position −570 (Fig. 3 shows a scheme). All four versions were conjugated into *Anabaena* sp. strain PCC 7120, and expression of the fusion was analyzed after nitrogen stepdown. As shown in Fig. 3B, after 6 h of nitrogen deprivation, all four strains showed strong, localized expression of the P_{hetC}::*gfp* fusion in individual cells, indicating that neither mutation of the critical elements of the promoter producing transcripts at position −571, that is, the NtcA binding box (with GTA changed to CAT; pCSAM162) or the −10 box (with TAN_3T changed to GGN_3G; pCSAM163), nor deletion of the sequences upstream from position −570 (pCSAM164) abolished localized induction of *hetC* in differentiating heterocysts. Localized induction of expression did not take place when the fusions were introduced into the *hetR* mutant strain 216 (Fig. 3C). These results corroborate the notion that HetR-dependent expression of *hetC* in (pro)heterocysts can take place from the promoter that produces transcripts starting at position −293. Taken together, these observations indicate that the sequence elements located downstream of position −571 are sufficient to provide HetR-dependent, localized, and increased expression of *hetC*.

Construction and analysis of strains bearing chromosomal duplications of the *hetC* promoter region. To further analyze the relationships between the two transcriptional start sites of the *hetC* gene, strains bearing a chromosomal duplication of the *hetC* promoter region were constructed. Two suicide plasmids were prepared that contained P_{hetC}::*gfp* translational fusions that included either the whole wild-type promoter region (up to position −980) or the deleted version of the promoter lacking sequences upstream from position −570. Integration of such plasmids into the *hetC* region of the chromosome resulted in partial duplication of sequences upstream from *hetC* so that two different versions of the *hetC* promoter directed expression of *gfp*, on the left side of the duplication, and of *hetC*, on the right side of the duplication. Both strains bear the wild-type *hetC* promoter on the left side, directing expression of the *gfp* gene, whereas expression of the *hetC* gene is under the control of a wild-type promoter region (strain CSAM157) or a short promoter region (strain CSAM160) (Fig. 4A). Transcription from position −293 in strains CSAM157 and CSAM160 was analyzed by primer extension. Oligonucleotides located outside of the repeated portion of the *hetC* region, GFP4 and HC4, were used in order to assess transcription from each copy of the promoter. Because it has been suggested that a functional HetC protein may be required for full activation of the expression of *hetC* (21), we designed this experimental setting so that transcription from the left side of the duplication was an internal control that helped interpretation in case any autoregulatory effects were observed. In order to preserve the stability of duplicated regions, all experiments involving strains CSAM157 and CSAM160, including nitrogen stepdown, were carried out in the presence of streptomycin and spectinomycin.

In strain CSAM157, transcriptions from position −293 on both sides of the duplication were similar (Fig. 4B). This was the expected result, as both copies of the *hetC* promoter are wild type in this strain. Both the *hetC* and the *gfp* transcripts were detected as early as 4 h after nitrogen stepdown, but their abundance was higher after 9 to 12 h (Fig. 4B). When transcription from the left-side promoter was analyzed in strain CSAM60, transcripts were readily detected after 4 hours of nitrogen stepdown, but the maximum accumulation was delayed with respect to strain CSAM157. Transcription from the right-side promoter in strain CSAM160 however, was altered, both with respect to the transcription observed in strain CSAM157 from the right-side promoter and with respect to transcription from the left side in both strains. In strain CSAM160, 5′ ends corresponding to the right side were detected at later time points than in the control strain, and the maximum accumulation was also delayed. The observation that, in strain CSAM160, transcription from position −293 was delayed in the right side of the duplication with respect to transcription from the left side indicates that such delay is due to the deletion introduced in the promoter and is not the result of the physiology of this strain being somehow altered. The fact that increased transcription from position −293 was readily observed in strain CSAM160 further confirmed that transcription from that position can take place in the absence of transcription from position −571 and from a promoter region lacking sequences upstream from position −570.

Strains CSAM157 and CSAM160 were able to grow on BG11 plates (containing nitrate) or $BG11_0$ plates (without nitrate) in the presence of streptomycin and spectinomycin (not shown). However, the observation of cultures subjected to nitrogen deficiency used for RNA isolation suggested that heterocyst differentiation was delayed in strain CSAM160. When subjected to nitrogen stepdown, filaments of control strain CSAM157 began to aggregate (a phenotype associated with deposition of heterocyst envelopes, which indicates progression of differentiation) at about 8 h after nitrogen stepdown,

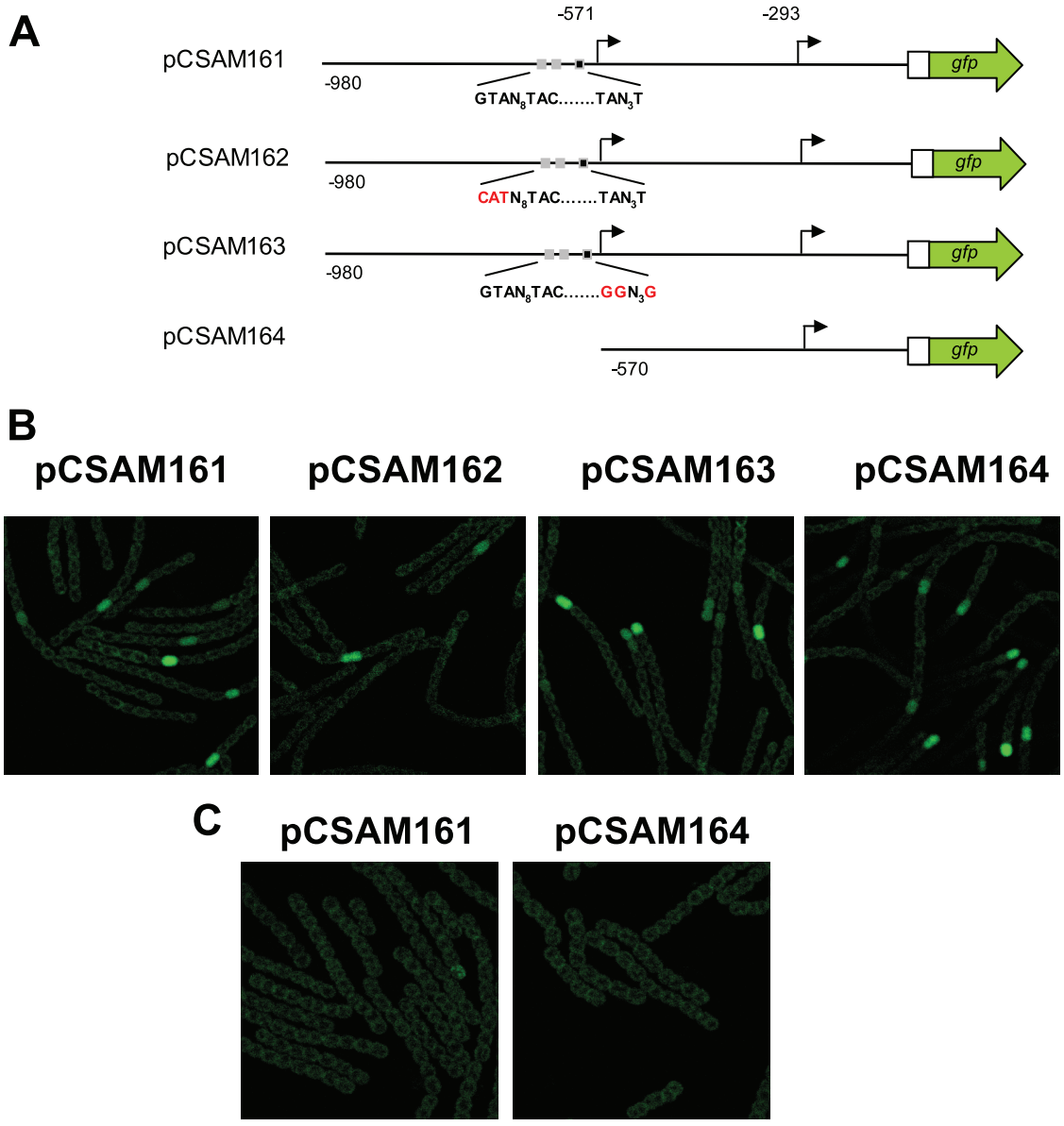

FIG. 3. Analysis of strains bearing P_{hetC}::*gfp* fusions in the replicative plasmids pCSAM161, pCSAM162, pCSAM163, and pCSAM164. (A) Scheme of the fusions between the *gfp* gene and the different versions of the *hetC* promoter present in each plasmid. Mutations introduced in the sequence are indicated in red. The positions of the −571 and −293 transcriptional start sites (black arrows) are indicated. The sizes of the *gfp* and *hetC* genes are not shown to scale. (B and C) Images of fluorescence corresponding to wild-type *Anabaena* sp. strain PCC 7120 (B) or *hetR* mutant strain 216 (C) bearing the plasmids indicated. The cells were grown in the presence of ammonium and incubated in the absence of combined nitrogen for 6 h.

and heterocysts that were stained with Alcian blue were readily observed by 10 h. At that time point, filaments of strain CSAM160 showed no aggregation, and no cells were stained with Alcian blue. Twenty-four hours after nitrogen stepdown, however, both CSAM157 and CSAM160 exhibited mature heterocysts, although the growth of strain CSAM160 in liquid medium under air/CO_2 bubbling was limited, showing a yellow-green color, and continued to be so up to 3 to 4 days later, when the appearances of cultures of the two strains became similar.

Transcriptional analysis of the NtcA-dependent *devB* gene. Because accumulation of *devBCA* transcripts, measured by Northern blotting using a *devB* probe (16) or as expression of *luxAB* fusions to *devA* (9), is dependent on HetR, we analyzed whether the initiation of transcription from the NtcA-activated transcriptional start site located at position −704 depended on HetR. The data shown in Fig. 5A indicate that the NtcA-dependent transcription of *devB* from position −704 took place in the two *hetR* mutants analyzed and was similar to that observed in the wild type up to 24 h after nitrogen stepdown. We performed primer extension experiments in order to identify other possible transcriptional start sites in the region upstream from *devB*. The results shown in Fig. 5B show the identification of a new transcriptional start site located at po-

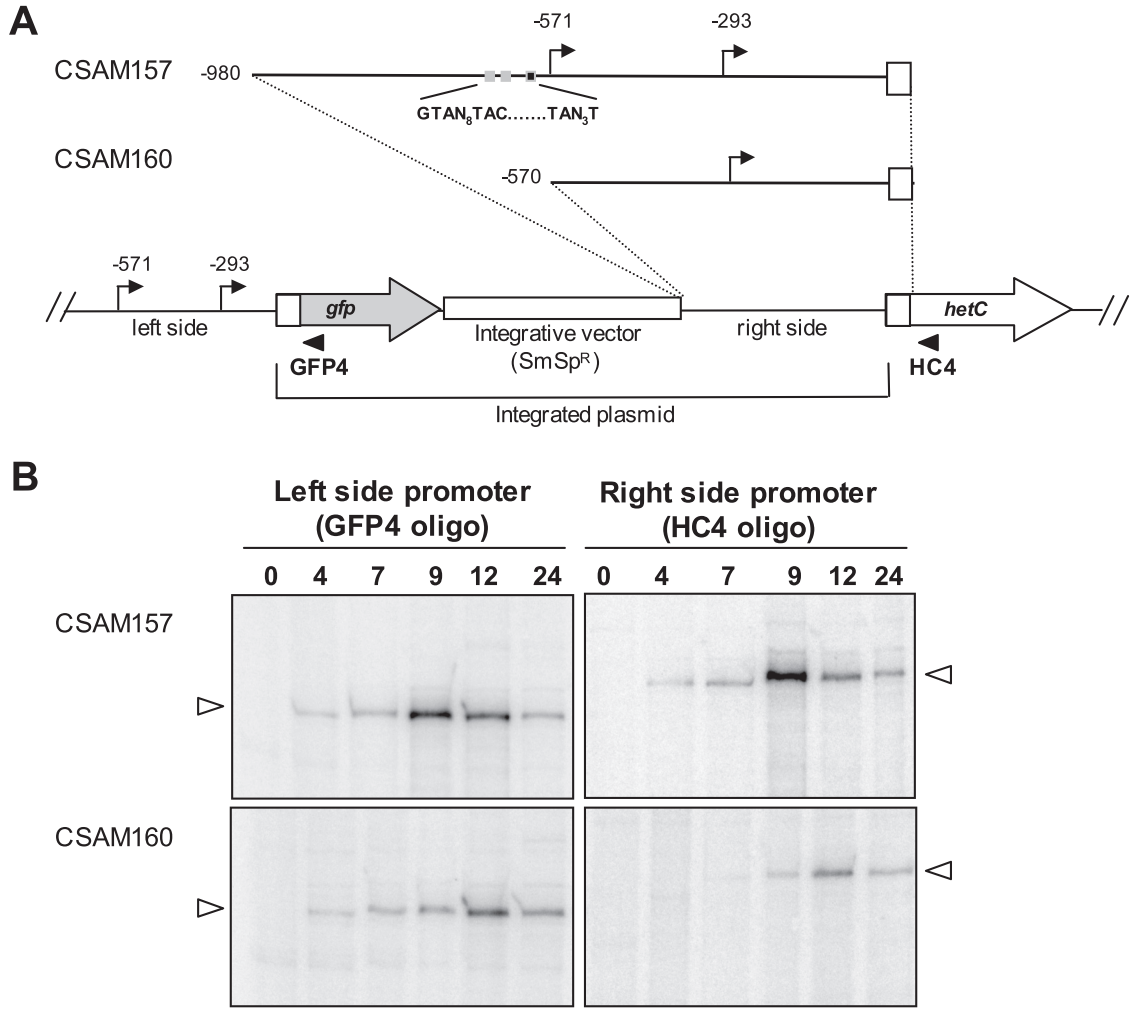

FIG. 4. Analysis of strains CSAM157 and CSAM160, bearing chromosomal duplications of the *hetC* promoter region. (A) Structure of the *hetC* chromosomal region in the strains bearing duplications of the *hetC* promoter after integration of plasmids bearing fusions between the *gfp* gene and two versions of the *hetC* promoter. The positions of the −571 and −293 transcriptional start sites (black arrows), as well as the features upstream from the −571 position (sequences corresponding to the NtcA box and −10 box), are indicated. The black triangles indicate the positions of relevant oligonucleotides used for primer extension assays. The sizes of the *gfp* and *hetC* genes are not shown to scale. (B) Primer extension analysis of transcription from position −293 in the two copies of the *hetC* promoter of strains CSAM157 and CSAM160. RNA was isolated from ammonium-grown filaments (lanes 0) or from ammonium-grown filaments incubated in the absence of combined nitrogen for the number of hours indicated above the lanes. For each RNA sample, oligonucleotides GFP4 (for transcripts originating at the left-side promoter) and HC4 (for transcripts originating at the right-side promoter) (the positions of the oligonucleotides are shown in panel A) were used as primers in parallel assays containing 25 μg of RNA. The white triangles point to transcripts originating at position −293. Note that products obtained with oligonucleotides GFP4 and HC4 have different lengths because of the different relative position of each oligonucleotide with respect to position −293.

sition −454, which was not observed in the *ntcA* strain CSE2 and was expressed at a very low, noninducible level in the *hetR* strain DR884a. Consistent with the accumulation of *devBCA* transcripts previously detected by Northern blotting (16), HetR-dependent expression from the −454 position was highest at 9 to 12 h after nitrogen stepdown. The same results were obtained using oligonucleotide DB15 (not shown).

DISCUSSION

The expression of many genes whose products act during the process of heterocyst differentiation depends on NtcA either directly, taking place from NtcA-activated promoters with a recognizable NtcA binding sequence, or indirectly through mechanisms that might involve other NtcA-dependent factors. HetR is also required for the expression of a number of heterocyst-specific genes, although the mechanism by which HetR exerts its positive effect remains unknown.

The *hetC* and *devB* genes, both of which are required for heterocyst differentiation, exhibit NtcA-dependent transcription, and consensus class II NtcA-activated promoters have been identified in the promoter regions of these two genes, producing transcripts with 5′ ends located at positions −571 (*hetC*) and −704 (*devB*) (16, 24). Transcriptional initiation from those promoters starts relatively early after nitrogen stepdown. In the case of *devB*, the transcript was readily observed

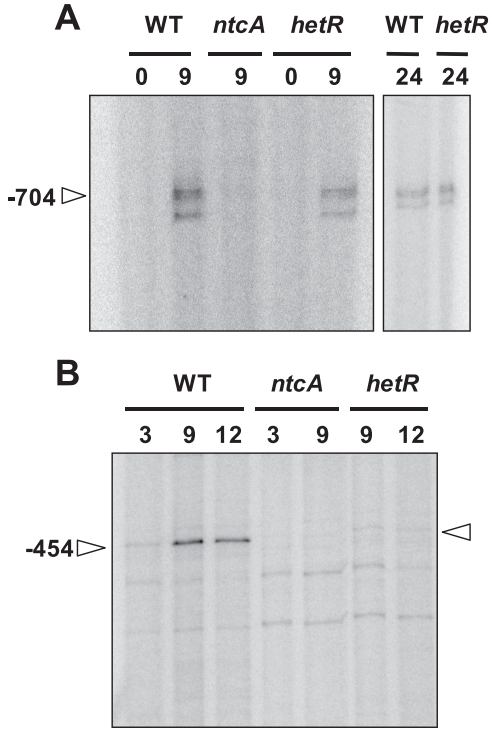

FIG. 5. Primer extension analysis of expression of *devB* in *Anabaena* sp. strain PCC 7120 and mutant strains CSE2 (*ntcA*), 216 (*hetR*), and DR884a (*hetR*). RNA was isolated from ammonium-grown cells (lanes 0) or from ammonium-grown cells incubated in the absence of combined nitrogen for the number of hours indicated above the lanes. Oligonucleotides OdevB7120 (A) and DB13 (B) were used as primers in assays containing 25 μg of RNA. The triangles point to the previously identified 5′ end corresponding to position −704 (16) (A) and to the 5′ end corresponding to position −454 (see the text) (B) with respect to the translational start site of the *devB* gene. WT, wild-type strain PCC 7120; *hetR*, strain DR884a, except for the 24-h sample in panel A, which is strain 216.

at 2 h after nitrogen stepdown (16), and in the case of *hetC*, the transcript was observed at 3 h, the earliest time point assayed (23, 24). According to primer extension assays, transcription from position −571 (*hetC*) continues at similar levels up to 24 h in the wild-type strain (24) and a *hetR* strain (23). Similar to the case for the promoter producing *hetC* transcripts that start at position −571, we have shown here that transcription of *devB* from position −704 does not require HetR (Fig. 5A). This observation suggests that, upon nitrogen stepdown, transcription from both promoters might take place in all the cells of the filament because, in both cases, it was observed in the *hetR* mutant, a strain showing no sign of differentiation. The pattern (HetR independent and, presumably, in all cells of the filament) and timing (relatively early after nitrogen stepdown) of expression from these two NtcA-activated promoters are similar to those of other consensus NtcA-dependent promoters whose induction does not require HetR, such as the P_1 promoter of the *glnA* gene (31) or the promoter of the *nrrA* gene (22). In fact, the NtcA consensus promoters located upstream of *hetC* (−571), *devB* (−704), and *nrrA* (−27) can be utilized in vitro in assays carried out with SigA-containing RNA polymerase (30). In such assays, transcription from each of these promoters requires both NtcA and 2-oxoglutarate, but no other factor (30). Activation of these promoters would not require the increased levels of NtcA that are present in proheterocysts at later stages of differentiation (25) as a result of HetR-dependent induction of the *ntcA* gene (23).

In addition to being dependent on NtcA, several reports indicate that the accumulation of the *hetC* and *devB* transcripts is also dependent on HetR (9, 16, 21). The Northern blot experiment shown here indicates that maximal accumulation of *hetC* transcripts peaks relatively late after nitrogen stepdown and is impaired in the *hetR* mutant. Thus, the regulation and timing of accumulation of both *hetC* and *devB* (16) transcripts differ from those observed for transcriptional initiation at positions −571 (*hetC*) and −704 (*devB*). This observation prompted us to further analyze the long promoter regions of both genes. In this work, we have shown that, in addition to the previously identified transcriptional start sites, both promoter regions contain a second promoter producing transcripts with a 5′ end located at positions −293 for *hetC* and −454 for *devB*. Expression from those positions is also NtcA dependent but, in contrast to expression from positions −571 and −704, requires HetR and appears to take place later during heterocyst differentiation. According to Fiedler et al. (16), expression from position −704 is already induced 2 h after N stepdown. The data shown here (Fig. 2B, 4B, and 5B) indicate that transcription from positions −293 (*hetC*) and −454 (*devB*) is already detectable after about 3 h of nitrogen stepdown but, consistent with Northern blot data (Fig. 2A) (16), peaks at a later stage of differentiation (around 9 to 12 h after nitrogen stepdown). Inspection of the sequences upstream from positions −293 for *hetC* and −454 for *devB* failed to detect any NtcA binding site that could be responsible for direct transcriptional activation by NtcA. The data presented here also indicate that, in the case of *hetC*, the sequences located between positions −570 and −293 are sufficient for regulated transcription of *hetC* from position −293, thus including a HetR-dependent promoter. We do not know whether this is also the case for transcription from the −454 position in the case of *devB*, but in any case, the distances between the upstream, HetR-independent TSP and the downstream, HetR-dependent TSP in the two genes are similar (278 nucleotides in the case of *hetC* versus 250 nucleotides in the case of *devB*).

Expression of *gfp* fused to fragments of the *hetC* or *devB* promoter regions (covering up to position −981 in the case of *hetC* and up to −988 in the case of *devB*) takes place mostly in heterocysts or proheterocysts (33, 36). One would expect that expression of the HetR-dependent promoters studied here for those two genes (producing the TSPs at −293 and −454, respectively) greatly contributes to localized expression of *gfp* fusions. According to our data, this is the case for the *hetC* gene. Both the whole promoter region of *hetC* (up to position −980) and a short version lacking the upstream promoter (down to position −570) produce (pro)heterocyst-localized expression of a translationally fused *gfp* gene.

Concerning the requirement for the *hetC* upstream region for diazotrophic growth, our results are consistent with previous observations by other researchers. In a previous report by Khudyakov and Wolk (21), three plasmids bearing *hetC* plus different lengths of upstream sequence were used to complement a *hetC* mutant. All three plasmids were able to

complement the mutation and support diazotrophic growth, producing apparently normal heterocysts with the same frequency and pattern as in the control wild-type strain. However, with N_2 as the nitrogen source, whereas exconjugants that had received larger plasmids grew with a dark-green color, those that received the smallest plasmid, bearing only 532 nucleotides of promoter sequence (and thus lacking the upstream promoter), grew with a yellow-green color, suggesting some degree of nitrogen limitation. The results shown in Fig. 4B also indicate that transcription from position −293 is impaired in strain CSAM160, in which the region directing expression of *hetC* does not include the promoter producing transcripts starting at position −571. Taken together, all these data indicate that, although transcription from −571 is not essential to support growth on N_2, both promoters are required for proper transcription of *hetC* and optimum diazotrophic growth.

Several genes whose products are involved in heterocyst differentiation exhibit long promoter regions with complex promoter arrangements. This is the case, for instance, for the genes encoding the two key regulators NtcA (23, 25–27) and HetR (6, 23). In both cases, there is a combination of constitutive and inducible promoters that results in strong localized expression in differentiating heterocysts. The results shown here indicate that the long promoter regions of the *hetC* and *devB* genes contain, in addition to the previously identified consensus NtcA-activated promoters, HetR-dependent promoters that might be indirectly regulated by NtcA. We can only speculate about the operation of such a promoter arrangement in vivo. According to data presented here, transcription from the two consensus class II promoters could be activated by NtcA relatively early after nitrogen stepdown, likely in all the cells of the filament. However, after a few hours of nitrogen stepdown, expression of the HetR-dependent downstream promoters, and accumulation of the corresponding transcripts, would be localized to specific cells differentiating into heterocysts. The organization of the promoter regions of these two genes, whose products are involved in heterocyst differentiation, adds complexity to the different types of promoters identified for genes included in the NtcA regulon.

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REFERENCES

1. **Aldea, M. R., K. Kumar, and J. W. Golden.** 2008. Heterocyst development and pattern formation, p. 75–90. *In* S. C. Winans and B. L. Bassler (ed.), Chemical communication among bacteria. ASM Press, Washington, DC.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 2007. Current protocols in molecular biology. Wiley-Interscience, New York, NY.
3. **Bensing, B. A., B. J. Meyer, and G. M. Dunny.** 1996. Sensitive detection of bacterial transcription initiation sites and differentiation from RNA processing sites in the pheromone-induced plasmid transfer system of *Enterococcus faecalis*. Proc. Natl. Acad. Sci. USA **93:**7794–7799.
4. **Black, T. A., Y. Cai, and C. P. Wolk.** 1993. Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. Mol. Microbiol. **9:**77–84.
5. **Buikema, W. J., and R. Haselkorn.** 1991. Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* 7120. Genes Dev. **5:**321–330.
6. **Buikema, W. J., and R. Haselkorn.** 2001. Expression of the *Anabaena hetR* gene from a copper-regulated promoter leads to heterocyst differentiation under repressing conditions. Proc. Natl. Acad. Sci. USA **98:**2729–2734.
7. **Buikema, W. J., and R. Haselkorn.** 1991. Isolation and complementation of nitrogen fixation mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **173:**1879–1885.
8. **Busby, S., and R. H. Ebright.** 1997. Transcription activation at class II CAP-dependent promoters. Mol. Microbiol. **23:**853–859.
9. **Cai, Y., and C. P. Wolk.** 1997. *Anabaena* sp. strain PCC 7120 responds to nitrogen deprivation with a cascade-like sequence of transcriptional activations. J. Bacteriol. **179:**267–271.
10. **Cai, Y., and C. P. Wolk.** 1990. Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. J. Bacteriol. **172:**3138–3145.
11. **Cormack, B. P., R. H. Valdivia, and S. Falkow.** 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene **173:**33–38.
12. **Ehira, S., and M. Ohmori.** 2006. NrrA directly regulates expression of *hetR* during heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **188:**8520–8525.
13. **Ehira, S., and M. Ohmori.** 2006. NrrA, a nitrogen-responsive response regulator facilitates heterocyst development in the cyanobacterium *Anabaena* sp. strain PCC 7120. Mol. Microbiol. **59:**1692–1703.
14. **Elhai, J., and C. P. Wolk.** 1988. Conjugal transfer of DNA to cyanobacteria. Methods Enzymol. **167:**747–754.
15. **Fiedler, G., M. Arnold, S. Hannus, and I. Maldener.** 1998. The DevBCA exporter is essential for envelope formation in heterocysts of the cyanobacterium *Anabaena* sp. strain PCC 7120. Mol. Microbiol. **27:**1193–1202.
16. **Fiedler, G., A. M. Muro-Pastor, E. Flores, and I. Maldener.** 2001. NtcA-dependent expression of the *devBCA* operon, encoding a heterocyst-specific ATP-binding cassette transporter in *Anabaena* spp. J. Bacteriol. **183:**3795–3799.
17. **Frías, J. E., E. Flores, and A. Herrero.** 1994. Requirement of the regulatory protein NtcA for the expression of nitrogen assimilation and heterocyst development genes in the cyanobacterium *Anabaena* sp. PCC 7120. Mol. Microbiol. **14:**823–832.
18. **Herrero, A., A. M. Muro-Pastor, and E. Flores.** 2001. Nitrogen control in cyanobacteria. J. Bacteriol. **183:**411–425.
19. **Herrero, A., A. M. Muro-Pastor, A. Valladares, and E. Flores.** 2004. Cellular differentiation and the NtcA transcription factor in filamentous cyanobacteria. FEMS Microbiol. Rev. **28:**469–487.
20. **Huang, X., Y. Dong, and J. Zhao.** 2004. HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS. Proc. Natl. Acad. Sci. USA **101:**4848–4853.
21. **Khudyakov, I., and C. P. Wolk.** 1997. *hetC*, a gene coding for a protein similar to bacterial ABC protein exporters, is involved in early regulation of heterocyst differentiation in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **179:**6971–6978.
22. **Muro-Pastor, A. M., E. Olmedo-Verd, and E. Flores.** 2006. All4312, an NtcA-regulated two-component response regulator in *Anabaena* sp. strain PCC 7120. FEMS Microbiol. Lett. **256:**171–177.
23. **Muro-Pastor, A. M., A. Valladares, E. Flores, and A. Herrero.** 2002. Mutual dependence of the expression of the cell differentiation regulatory protein HetR and the global nitrogen regulator NtcA during heterocyst development. Mol. Microbiol. **44:**1377–1385.
24. **Muro-Pastor, A. M., A. Valladares, E. Flores, and A. Herrero.** 1999. The *hetC* gene is a direct target of the NtcA transcriptional regulator in cyanobacterial heterocyst development. J. Bacteriol. **181:**6664–6669.
25. **Olmedo-Verd, E., A. M. Muro-Pastor, E. Flores, and A. Herrero.** 2006. Localized induction of the *ntcA* regulatory gene in developing heterocysts of *Anabaena* sp. strain PCC 7120. J. Bacteriol. **188:**6694–6699.
26. **Olmedo-Verd, E., A. Valladares, E. Flores, A. Herrero, and A. M. Muro-Pastor.** 2008. Role of two NtcA-binding sites in the complex *ntcA* gene promoter of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **190:**7584–7590.
27. **Ramasubramanian, T. S., T.-F. Wei, A. K. Oldham, and J. W. Golden.** 1996. Transcription of the *Anabaena* sp. strain PCC 7120 *ntcA* gene: multiple transcripts and NtcA binding. J. Bacteriol. **178:**922–926.
28. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Generic assignments, strain stories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.
29. **Shi, Y., W. Zhao, W. Zhang, Z. Ye, and J. Zhao.** 2006. Regulation of intracellular free calcium concentration during heterocyst differentiation by HetR and NtcA in *Anabaena* sp. PCC 7120. Proc. Natl. Acad. Sci. USA **103:**11334–11339.
30. **Valladares, A., E. Flores, and A. Herrero.** 2008. Transcription activation by NtcA and 2-oxoglutarate of three genes involved in heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **190:**6126–6133.
31. **Valladares, A., A. M. Muro-Pastor, A. Herrero, and E. Flores.** 2004. The

NtcA-dependent P_1 promoter is utilized for *glnA* expression in N_2-fixing heterocysts of *Anabaena* sp. strain PCC 7120. J. Bacteriol. **186:**7337–7343.
32. **Vioque, A.** 1997. The RNase P RNA from cyanobacteria: short tandemly repeated repetitive (STRR) sequences are present within the RNase P RNA gene in heterocyst-forming cyanobacteria. Nucleic Acids Res. **25:**3471–3477.
33. **Wang, Y., and X. Xu.** 2005. Regulation by *hetC* of genes required for heterocyst differentiation and cell division in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **187:**8489–8493.
34. **Wei, T.-F., T. S. Ramasubramanian, and J. W. Golden.** 1994. *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. J. Bacteriol. **176:**4473–4482.
35. **Xu, X., J. Elhai, and C. P. Wolk.** 2008. Transcriptional and developmental responses by *Anabaena* to deprivation of fixed nitrogen, p. 383–422. *In* A. Herrero and E. Flores (ed.), The cyanobacteria. Molecular biology, genomics and evolution. Caister Academic Press, Norfolk, United Kingdom.
36. **Xu, X., and C. P. Wolk.** 2001. Role for *hetC* in the transition to a nondividing state during heterocyst differentiation in *Anabaena* sp. J. Bacteriol. **183:**393–396.
37. **Zhao, J., and C. P. Wolk.** 2008. Developmental biology of heterocysts, 2006, p. 397–418. *In* D. E. Whitworth (ed.), Myxobacteria: multicellularity and differentiation. ASM Press, Washington, DC.
38. **Zhou, R., X. Wei, N. Jiang, H. Li, Y. Dong, K.-L. Hsi, and J. Zhao.** 1998. Evidence that HetR protein is an unusual serine-type protease. Proc. Natl. Acad. Sci. USA **95:**4959–4963.