Mutual dependence of the expression of the cell differentiation regulatory protein HetR and the global nitrogen regulator NtcA during heterocyst development

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Summary

Heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120 depends on both the global nitrogen regulator NtcA and the cell differentiation regulatory protein HetR, and induction of *hetR* upon nitrogen step-down depends on NtcA. The use of two of the four transcription start points (tsps) described for the *hetR* gene (those located at positions -728 and -271) was found to be dependent on NtcA, and the use of the tsp located at position -271 was also dependent on HetR. Thus, autoregulation of *hetR* could take place via the activation of transcription from this tsp. Expression of *ntcA* in nitrogen-fixing cultures was higher than in cells growing in the presence of ammonium or nitrate, and high expression of *ntcA* under nitrogen deficiency resulted from an increased use of tsps located at positions -180 and -49. The induction of the use of these tsps did not take place in *ntcA* or *hetR* mutant strains. These results indicate a mutual dependency in the induction of the regulatory genes *hetR* and *ntcA* that takes place in response to nitrogen step-down in *Anabaena* cells. Expression of the *hetC* gene, which is also involved in the early steps of heterocyst differentiation, from its NtcA-dependent tsp was however not dependent on HetR.

Introduction

Cyanobacteria are a widely distributed group of phototrophic bacteria that carry out oxygenic photosynthesis. Cyanobacteria are able to use different nitrogen sources, including nitrate and ammonium, and many can also fix atmospheric nitrogen. Ammonium is used in preference over nitrate, which is used in preference over dinitrogen (Flores and Herrero, 1994; Herrero *et al.,* 2001). At the molecular level, this possibility of choice is based on a nutritional repression exerted by ammonium on the expression of genes involved in the assimilation of alternative nitrogen sources, including atmospheric nitrogen, and *ntcA* has been identified as a gene that encodes a transcriptional regulator operating global nitrogen control that is widespread in cyanobacteria (Frías *et al*., 1993; Luque *et al*., 1994; Herrero *et al.,* 2001). The NtcA protein belongs to the CRP family of bacterial regulators and bears close to its C-terminal end a helix-turn-helix motif for interaction with DNA (Vega-Palas *et al*., 1992). NtcA binds to specific sites in the promoter region of regulated genes involved in nitrogen assimilation and activates their expression in response to ammonium withdrawal (Luque *et al*., 1994). The structure of the consensus cyanobacterial NtcA-activated promoter comprises a -10 box in the form TAN_3T and an NtcA-binding site containing the sequence signature $GTAN_8TAC$ that is located 20-23 nucleotides upstream from the -10 box and appears to substitute for the -35 box that would be present in promoters similar to the canonical *E. coli* σ^{70} promoters (Luque *et al*., 1994; Herrero *et al*., 2001).

The ability to fix atmospheric nitrogen depends on the oxygen-sensitive enzyme nitrogenase. Some filamentous cyanobacteria are able to differentiate, under conditions of aerobiosis and combined nitrogen deprivation, cells specialized in nitrogen fixation called heterocysts that develop from vegetative cells located at semiregular intervals in the filament (Buikema and Haselkorn, 1993; Wolk, 2000).

Heterocysts provide the microaerobic environment required for nitrogenase activity. The differentiation process begins shortly after nitrogen deprivation and, under our experimental conditions, requires about 24 h to complete. Heterocysts differ from vegetative cells in many structural and functional features that turn them into efficient factories for nitrogen fixation. At the molecular level, the differential physiology of the heterocyst is supported by a pattern of gene expression that largely differs from that taking place in vegetative cells (Buikema and Haselkorn, 1993; Wolk, 2000).

Heterocyst development requires the product of the regulatory gene *hetR* (Buikema and Haselkorn, 1991; Black *et al*., 1993), which is not required for growth using combined nitrogen and whose expression is induced shortly after nitrogen deprivation. Induction of the expression of *hetR* does not take place in a *ntcA* mutant (Frías *et al*., 1994). The NtcA protein is required for heterocyst development (Frías *et al*., 1994; Wei *et al*., 1994) as well as for the function of mature heterocysts (Herrero *et al*., 2001) and appears to link nitrogen deficiency and heterocyst differentiation in the context of a hierarchy of the use of different nitrogen sources. Another early-acting heterocyst development gene is *hetC*, whose product shows homology to ATP-binding cassette (ABC) transporters (Khudyakov and Wolk, 1997).

Primer extension analysis has been carried out to define the transcription start points (tsps) for the *ntcA*, *hetR*, and *hetC* genes in *Anabaena* sp. strain PCC 7120. Three extension products that localize RNA 5' ends at positions -49, -180, and -190 with respect to the *ntcA* translation start have been described, and DNA fragments carrying the regions around the -49 and -180/-190 sites have been found to exhibit promoter activity tested with a *lacZ* reporter, indicating the presence of at least two different promoters in the *ntcA* upstream region (Ramasubramaniam *et al*., 1996). Additionally, at least the -49 site is preceded by a putative -10 promoter hexamer. Four extension products have been reported for *hetR* that localize RNA 5' ends at

-184, -271, -696, and -728 nucleotides with respect to the translation start of this gene (Buikema and Haselkorn, 2001). Although no independent evidence for promoter activity upstream from these sites has been presented, the fact that they may correspond to *hetR* transcripts of 1.4/1.5 and 1.9 kb detected by Northern analysis (Buikema and Haselkorn, 1991) suggests that those 5' ends do not originate from primer extension artifacts. Additionally, because the long (1.9 kb) and short (1.4/1.5 kb) *hetR* transcripts show different expression patterns, they likely originate from different promoters. Scrutiny of the nucleotide sequences upstream from those RNA 5' ends shows that the four of them are preceded, with a spacing of 6 or 8 nt, by putative -10 promoter hexamers, consistent with the possibility that they represent true tsps. Finally, the *hetC* gene has been found to be expressed from an NtcA-activated promoter that generates a tsp at -571 with respect to the translation start of the gene (Muro-Pastor *et al.*, 1999).

In an attempt to determine the relationships between the products of *hetR* and *ntcA* during the triggering of heterocyst differentiation, we have studied the accumulation of their transcripts in strains with different genetic backgrounds (i.e., wild type, *ntcA*, *hetR*). We have also analyzed the use of transcription start sites for both genes in the *ntcA* and *hetR* strains as well as the effect of *hetR* inactivation on the transcription of *hetC*.

Results

Levels of ntcA *expression*

The NtcA protein is dispensable for growth using ammonium as a nitrogen source. However, it is necessary for growth using nitrate or atmospheric nitrogen. Fig. 1 shows that cultures of *Anabaena* sp. strain PCC 7120 growing under different nitrogen regimes exhibited different levels of the *ntcA* mRNA. As previously

described (Wei *et al*., 1994; Ramasubramanian *et al*., 1996), *ntcA* was expressed as several transcripts in the range 0.8-1.4 kb. The *ntcA* expression level was similar in cells growing in the presence of ammonium or nitrate. However, when the cells were grown under diazotrophic conditions, *ntcA* was expressed at a higher level (2.6-fold the level of transcripts found in ammonium-containing medium for the experiment shown in Fig. 1). This observation might suggest that the level of *ntcA* transcription required for growth under diazotrophic conditions is higher than that necessary for growth on nitrate. Although in both cases there is a strict requirement for NtcA, diazotrophic growth of *Anabaena* filaments involves the development of nitrogen-fixing heterocysts.

Time-course of induction of ntcA *and* hetR *in response to nitrogen deprivation*

In *Anabaena* sp. strain PCC 7120, expression of *hetR* is induced shortly after nitrogen deprivation (Buikema and Haselkorn, 1991; Black *et al*., 1993) in an NtcA-dependent manner (Frías *et al*., 1994). Similar results have been described for the symbiotically competent *Nostoc punctiforme* strain ATCC 29133 (Wong and Meeks, 2001). The precise time course of the induction of expression of *ntcA* and *hetR* depends on the experimental conditions, so that results from different laboratories may not be strictly comparable. We have carried out Northern experiments in order to compare the time course of induction of the two genes, *ntcA* and *hetR*, when the cells are subjected to nitrogen deprivation. Under our experimental conditions, induction of expression of *hetR* took place, in wild type cells, after less than three hours of nitrogen deficiency, and expression was highest between 4.5 and, at least, 9 h (Fig. 2A, upper panels, left side in both cases). As expected (Frías *et al*., 1994), there was no increase in the abundance of the *hetR* transcripts in response to nitrogen deprivation in an *ntcA* background (strain CSE2). Thus, in the *ntcA* mutant, there was

no increase in the abundance of the 1.4/1.5-kb *hetR* transcripts already observed in the presence of ammonium and essentially no 1.9-kb transcripts appeared (Fig. 2A, upper right panel, right side, and Fig. 2C). Induction of *ntcA* was observed to start sometime between 4.5 and 6 h of nitrogen deficiency (Fig. 2A, central panels, left side in both cases), a time frame in which *hetR* expression was already induced. Thus, the initial induction of expression of *hetR* in response to nitrogen deprivation precedes in time that of *ntcA*. This observation suggests that the level of *ntcA* transcription that takes place in the presence of ammonium, a condition in which NtcA is not required for growth, is sufficient to induce *hetR* in response to nitrogen deficiency.

The results shown in Fig. 2A (left central panel, right side) and Fig. 2B additionally indicate that the induction of expression of *ntcA* in response to nitrogen deficiency depends on HetR, since it does not take place in a *hetR* mutant background (strain 216). This observation, together with the results shown in Fig. 1, indicates that the increased expression of *ntcA* observed in diazotrophic cultures is not required for the initial stages of heterocyst development but rather for later stages once the induction of expression of the cell differentiation regulatory gene *hetR* has taken place.

hetR *promoters*

Both the *hetR* (Buikema and Haselkorn 2001) and *ntcA* (Ramasubramanian *et al*., 1996) genes are transcribed from complex promoters with multiple transcription start sites. To determine the regulation of expression from each of those tsps, primer extension experiments were carried out with RNA isolated from wild type *Anabaena* sp. strain PCC 7120, the *ntcA* mutant CSE2 (Frías *et al*., 1994), and two *hetR* strains, insertional mutant DR884a (Black *et al*., 1993) and strain 216, which bears a *hetR*

point mutation, S179A (Buikema and Haselkorn 1991). The four putative tsps described for the *hetR* gene in *Anabaena* sp. strain PCC 7120 have been reported to be nitrogen regulated (Buikema and Haselkorn, 2001). To analyze the *hetR* tsps, we isolated RNA from wild type and mutant cells grown in the presence of ammonium or grown with ammonium and subjected to nitrogen starvation for 6 h. Results shown in Fig. 3C, D indicate that, under our experimental conditions, the use of the initiation site located at position -184 was not nitrogen-regulated, since this site was used in the wild type both in cells grown in ammonium-containing media and in cells subjected to nitrogen deprivation for 6 h, as well as in the *ntcA* and *hetR* mutant strains. However, in the wild type, the use of the other three initiation sites (located at -728, -696, and -271) was activated upon nitrogen step-down (Fig. 3).

The tsp located at position -271 was not used in the *ntcA* strain CSE2 (Fig. 3C), indicating that its use is dependent on the nitrogen regulator NtcA. This inducible tsp is also dependent on HetR, since it was very weakly used in any of the two *hetR* strains, the insertional mutant (strain DR884a) and the point mutant (strain 216) (Fig. 3D). Thus, in this regard, both *hetR* mutations have the same effect indicating that the use of the tsp located at position -271 requires a functional HetR protein, consistent with the autoregulatory character of *hetR* (Black *et al*., 1993; Buikema and Haselkorn, 2001). Induction of the use of the tsp located at position -696 was independent of both NtcA and HetR, whereas the induction of the tsp located at position -728 was severely reduced in the *ntcA* mutant but unaffected by mutations in *hetR* (Fig. 3A, B). The sequences of the region upstream from the two NtcA-regulated tsps (-728 and -271) do not match the sequences found in canonical NtcA-activated promoters, and band-shift assays containing purified NtcA protein and fragments corresponding to the promoter region of *hetR* have failed to show any binding of NtcA to those promoters *in vitro* (not shown). Thus, the NtcA dependence

of the utilization of both tsps may be indirect, that is, mediated by other protein(s) that could, in turn, be regulated by NtcA. The *hetR* transcript found in the presence of ammonium (Fig. 2A, upper right panel) originates, most likely, from the tsp located at position -184, which is constitutive. Expression from the -271 tsp, which is dependent on HetR and originates the 1.5 kb transcript, would account for the autoregulatory character of *hetR* (Black *et al*., 1993; Buikema and Haselkorn, 2001). Finally, the large, 1.9-kb transcript, which is almost not induced in the *ntcA* mutant, may have its origin mostly at the NtcA-dependent -728 tsp.

ntcA *promoters*

In order to study, at the level of transcription initiation, the effects of *hetR* and *ntcA* mutations on *ntcA* expression, primer extension experiments with RNA isolated from cells subjected to different nitrogen regimes were carried out (Fig. 4). Under our experimental conditions, extension products ending at positions -49, -136 and -180 were found. We only detected a very faint band that could correspond to the tsp previously found at position -190 (Ramasubramanian *et al*., 1996). The extension product whose 5' end located at position -136 appeared to be constitutive, being used in nitrate- or ammonium-containing media as well as in cells subjected to nitrogen deficiency or in diazotrophic cultures, and it was also observed in the *hetR* and the *ntcA* mutant backgrounds (Fig. 4A). Centered 10.5 nucleotides upstream of the -136 site, a putative -10 promoter box (TATCAT) is present, supporting the assignation of the -136 site as an additional transcription start for the *ntcA* gene.

The tsp located at position -49 was also observed under all conditions, but its use was induced under nitrogen deprivation (Fig. 4A; see also Ramasubramanian *et al*., 1996). The putative tsp located at position -180, on the contrary, was only used in the absence of combined nitrogen and exhibited a strong, transient induction

between 6 and 12 h of nitrogen deprivation. Induction of the use of the -49 and -180 tsps was dependent on both NtcA and HetR (Fig. 4A). This induction may be responsible for the increase in the abundance of *ntcA* transcripts observed at about 4.5-6 h of nitrogen deprivation, an increase that depends on HetR (Fig. 2). Quantification of the signals in the Northern blot shown in Fig. 2A (central left panel) indicates that transient induction of *ntcA* can lead to levels of *ntcA* transcript 5-fold higher (at 9 h of nitrogen deprivation) than the level observed in ammonium-containing medium (Fig. 2B), in contrast to the levels of *ntcA* transcript observed in diazotrophic cultures (2.6-fold higher than the level observed in cells grown in ammonium-containing medium, see Fig. 1). Expression of *ntcA* in heterocysts seems to take place mostly from the tsp located at position -49, although there is also some expression from the -136 and -180 tsps (Fig. 4B).

The results shown in Fig. 4 indicate that HetR exerts a positive effect on *ntcA* transcription via the induction of both the -49 and the -180 tsps. As shown in Fig. 1, the level of expression of *ntcA* in cells growing diazotrophically is higher than that found in cells growing in the presence of nitrate. Since utilization of nitrate requires NtcA protein and the *hetR* mutants can use nitrate as a nitrogen source, the level of *ntcA* expression found in *hetR* mutants must be sufficient to sustain growth using nitrate as a nitrogen source.

Expression of the hetC *gene in a* hetR *mutant strain*

We have analyzed, at the level of transcription initiation, the effect of a *hetR* mutation on the expression of *hetC*, another gene acting early in the process of heterocyst development (Khudyakov and Wolk, 1997) whose expression depends on NtcA (Muro-Pastor *et al*., 1999). Transcription initiation from the nitrogen-regulated tsp located at position -571 (Muro-Pastor *et al*., 1999) takes place in the *hetR* mutant

strain, thus indicating that the use of this NtcA-dependent tsp is HetR-independent (Fig. 5).

Discussion

NtcA, the transcriptional factor that operates global nitrogen control in cyanobacteria is required for growth of *Anabaena* sp. strain PCC 7120 using either nitrate or atmospheric dinitrogen. Growth with dinitrogen involves the differentiation of heterocysts, which does not take place in an *ntcA* mutant. Heterocyst development also requires the product of the *hetR* gene, which is dispensable for growth in the presence of nitrogen sources other than dinitrogen. Induction of *hetR* in response to nitrogen deprivation depends on NtcA both in *Anabaena* sp. strain PCC 7120 (Frías *et al*., 1994) and in *Nostoc punctiforme* (Wong and Meeks, 2001). In *Anabaena* sp. strain PCC 7120, the analysis of *hetR* expression at the level of transcription initiation indicates that the use of two of the four putative tsps described for this gene, those located at -271 and -728, depends on NtcA. However, no nucleotide sequence matching the consensus for NtcA-activated promoters can be found upstream from those tsps, making the molecular basis of this dependence unknown. Expression from the tsp located at position -271 is also dependent on HetR, suggesting that the autoregulation of *hetR* (Black *et al*., 1993) might be operated via expression from this tsp. It has been described that expression of the *hetR*::*luxAB* fusion in strain DR884a is not increased upon nitrogen deprivation (Black *et al*., 1993). However, in the case of mutant strain 216, a certain level of induction takes place, as determined by Northern analysis (Fig. 2A; see also Buikema and Haselkorn, 2001) and Western detection of HetR protein (Dong *et al*., 2000). Our data indicate that both *hetR* strains behave identically at the level of transcription initiation and mutation of *hetR* significantly affects the expression from the -271 tsp only (Fig.3).

Expression of *ntcA* is higher under diazotrophic conditions than in nitrogen-replete medium. The NtcA protein is not only required for heterocyst differentiation (Frías *et al*., 1994; Wei *et al*., 1994), but also for expression of genes whose products are critical for heterocyst function. The *petH* gene, encoding ferredoxin:$NADP^+$ reductase, is expressed in heterocysts mostly from an NtcA-activated promoter (Valladares *et al*., 1999) and, similarly, the *glnA* gene, encoding glutamine synthetase, is expressed in heterocysts from its NtcA-dependent promoter (our unpublished results). Expression of *ntcA* is induced upon nitrogen deprivation. Such induction involves increased transcription from the tsps located at positions -49, a tsp that is also used, although at a lower level, in combined nitrogen-containing medium, and -180, a tsp that is transiently expressed only during heterocyst development. Expression from the -49 tsp seems to be directly regulated by NtcA. Two putative NtcA binding sites can be identified upstream from this tsp. Neither of them is centered at about 40.5 nucleotides upstream from the tsp, the position of the NtcA binding site in most NtcA-activated promoters characterized so far (Luque *et al*., 1994; Herrero *et al*., 2001), but one of them, centered at 94.5 nucleotides upstream from the tsp, has been footprinted using partially purified NtcA protein from *Anabaena* sp. strain PCC 7120 (Ramasubramanian *et al.,* 1996). We have carried out *in vitro* footprinting experiments with purified histidine-tagged NtcA that essentially confirm the results obtained by Ramasubramanian *et al.* (not shown). Since the footprinted NtcA binding site is much further upstream from the tsp than usual, this promoter could correspond to a different class of NtcA activated promoters resembling the class I CRP-activated promoters of *E. coli* (see also P*nblA*-2, Luque *et al*., 2001). On the other hand, this binding site overlaps the putative -10 box for the constitutively used tsp located at position -136. This observation might suggest a negative effect of NtcA on the operation of this promoter that could be manifest in the presence of high

concentrations of NtcA. The -49 tsp is also the major tsp used in heterocysts, further suggesting an active role for NtcA in transcription in heterocysts. Nevertheless, *ntcA*-containing clones able to complement *in trans* the *ntcA* mutation in *Anabaena* could only support diazotrophic growth if the *ntcA* upstream region included the -180 and -190 sites, indicating that expression from those tsps is critical for growth on dinitrogen, whereas clones whose upstream region contained only the -49 site were able to sustain growth on nitrate but not in nitrogen-free medium (Ramasubramanian *et al*., 1996). Consistently, our experiments indicate that transcription from the -180 tsp is, at least in part, responsible for the high level of *ntcA* expression that takes place only under combined nitrogen deprivation. It seems that activation of the use of the -180 tsp is required at an intermediate stage during heterocyst development, whereas the -49 tsp is the one being used in mature heterocysts (see also Ramasubramanian *et al*., 1996). As was also the case for the NtcA-dependent promoters of the *hetR* gene, no consensus site for NtcA binding can be identified upstream from the -180 tsp. Thus, the participation of additional regulators whose expression is, in turn, directly regulated by NtcA must be taken into consideration.

The increased level of *ntcA* expression observed during the process of heterocyst differentiation depends on the development-specific *hetR* gene. The observation that the induction of *ntcA* peaks later than the induction of *hetR* suggests that high levels of NtcA are not necessary to induce *hetR* and to trigger heterocyst development but rather to continue the process once it has started. The fact that activation of expression of *ntcA* during heterocyst development depends on HetR could explain why expression of some NtcA-activated, late-acting, heterocyst development genes is also dependent on HetR. Such dependence could conceivably be operated via NtcA. This could be the case for the *devBCA* operon, whose expression is HetR-dependent (Cai and Wolk, 1997) and directly regulated by NtcA,

as indicated by its expression from a consensus cyanobacterial NtcA-activated promoter to which NtcA has been found to bind (Fiedler *et al*., 2001). Also, the expression of the *devH* gene depends on NtcA and HetR (Hebbar and Curtis, 2000). In contrast, expression of NtcA-dependent, early acting genes such as *hetC* (Khudyakov and Wolk, 1997; Muro-Pastor *et al*., 1999), would be expected to be HetR independent, at least at the level of transcription initiation. Results shown in Fig. 5 indicate that this is actually the case for *hetC*, since expression from the NtcA-activated promoter that originates the -571 tsp is not dependent on HetR. Additionally, expression of NtcA-activated genes not involved in heterocyst development but required for growth on nitrate would not require the HetR-dependent increased levels of NtcA. HetR-dependent induction of *ntcA* appears therefore to represent a mechanism to ensure that, during heterocyst development, the levels of NtcA are high. Expression of *hetR* is induced in specific cells after nitrogen step-down (Black *et al*., 1993). Whether the high level of expression of *ntcA* under diazotrophic conditions is due to expression in the developing heterocysts, as suggested by its HetR dependency, or in the whole filament remains to be determined.

Experimental procedures

Organisms and culture conditions

This study was carried out with the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 and three Het^- derivatives, strain CSE2, an insertional mutant of the *ntcA* gene (Frías *et al*., 1994), strain DR884a, an insertional mutant of the *hetR* gene (Black *et al*., 1993), and strain 216, which bears a point mutation in the *hetR* gene (Buikema and Haselkorn, 1991). Cells were grown in BG11 liquid medium (Rippka *et al*., 1976), $BG11_0$ medium (medium BG11 without $NaNO_3$) or $BG11_0$ medium

containing 8 mM NH_4Cl and 16 mM TES-NaOH buffer (pH 7.5). All cultures were supplemented with 10 mM of $NaHCO_3$ and bubbled with a mixture of CO_2 and air (1:99 vol/vol). Strain CSE2 was grown in the presence of 2 $\mu g \cdot ml^{-1}$ of streptomycin and 2 $\mu g \cdot ml^{-1}$ of spectinomycin. Strain DR884a was grown in the presence of 5 $\mu g \cdot ml^{-1}$ of neomycin. Cultures for heterocyst isolation were grown in ammonium-containing medium until they reached the exponential phase. Cells were then washed with, and resuspended in, nitrogen-free medium ($BG11_0$) and further incubated until mature heterocysts were observed (19 h in the case of the experiment shown in Fig. 4B). Heterocysts were isolated as described (Golden *et al*., 1991).

RNA methods

For RNA isolation, cells growing exponentially in NH_4Cl-containing medium were harvested at room temperature and either used directly or washed with, and resuspended in, $BG11_0$ medium and further incubated under culture conditions for the number of hours indicated in each experiment. Alternatively, cells growing exponentially in N_2, ammonium or nitrate were used. RNA from whole filaments or from isolated heterocysts was isolated in the presence of ribonucleoside-vanadyl complex according to Ausubel *et al*. (2001), with modifications. No CsCl gradient was performed. After phenol extraction and ethanol precipitation, samples were treated with RNase-free DNase and ethanol-precipitated again.

Primer extension analysis of the *hetR* transcripts was carried out as previously described (Muro-Pastor *et al*., 1999) with ^{32}P-labeled oligonucleotides HR2 (complementary to positions -296 to -319 relative to the translation start of *hetR*), HR3 (complementary to positions -63 to -82 relative to the translation start of *hetR*), HR7 (complementary to positions -45 to -64 relative to the translation start of *hetR*), and hetRPEX3 (complementary to positions -604 to -622 relative to the translation

start of *hetR*; Buikema and Haselkorn, 2001). In the case of the *ntcA* gene, the oligonucleotides used were NA1 (complementary to positions +59 to +40 relative to the translation start of *ntcA*) and NA7 (complementary to positions +14 to -4 relative to the translation start of *ntcA*). In the case of the *hetC* gene, the oligonucleotide used was HC2 (complementary to positions -411 to -431 relative to the translation start of *hetC*).

Northern blot analysis was carried out as described (Muro-Pastor *et al*., 1999). The *hetR* probe was a 703-bp *Hae* II fragment containing most of the *hetR* gene (Buikema and Haselkorn, 1991). The *ntcA* probe was a 690-bp *Nco* I-*Sal* I fragment from pCSAM61 (a plasmid containing the whole *Anabaena ntcA* gene cloned between the *Nco* I and *Bam*H I sites of expression vector pTrc99A [Amersham Pharmacia]). Images of radioactive filters and gels were obtained and quantified with a Cyclone storage phosphor system and OptiQuant image analysis software (Packard).

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Figure legends

Fig. 1. Northern blot analysis of expression of the *ntcA* gene in *Anabaena* sp. strain PCC 7120. RNA was isolated from cells grown in the presence of ammonium (lane NH_4^+) or nitrate (lane NO_3^-) or from diazotrophic cultures (lane N_2). Samples contained 70 μg of RNA. Hybridizations to a *ntcA* probe (upper panel) or to a *rnpB* (Vioque, 1997) probe as loading and transfer control (lower panel) were performed. Quantification of the *ntcA* transcripts was normalized using the *rnpB* signal, and the transcript level was divided by the value for the NH_4^+- grown culture (arbitrarily set to 1.0). Values obtained were 2.6 (N_2) and 1.2 (NO_3^-). Arrowheads point to the different transcripts described for the *ntcA* gene.

Fig. 2. Northern blot analysis of expression of the *ntcA* and *hetR* genes in *Anabaena* sp. strain PCC 7120, the *ntcA* strain CSE2 and the *hetR* strain 216. RNA was isolated from ammonium-grown cells or from ammonium-grown cells subjected to nitrogen deficiency for the number of hours indicated in each case. Samples in the filters shown in the left and right panels correspond to different experiments including the wild type strain in both cases. Samples contained 50 μg of RNA. (A) The filters were hybridized to a *hetR* probe (upper panels), an *ntcA* probe (central panels) or an *rnpB* probe (Vioque, 1997) (lower panels). Note that the absence of signal in the right side of central right panel (strain CSE2, *ntcA* probe) can be due to disruption of the *ntcA* gene in strain CSE2 that might render the *ntcA* transcript unstable or not detectable with the *ntcA* probe used. (B) Relative abundance of the *ntcA* transcript in the wild type and the *hetR* mutant. Signals (entire lane, including the smear) in the central left panel of Fig. 2A were quantified and normalized using the *rnpB* signal (lower left panel). The highest value was arbitrarily set to 100. (C) Relative abundance of the *hetR* transcript in the wild type and the *ntcA* mutant. Signals (entire

lane, including the smear) in the upper right panel of Fig. 2A were quantified and normalized using the *rnpB* signal (lower right panel). The highest value was arbitrarily set to 100. WT, wild type strain PCC 7120. Arrowheads point to the different transcripts described for the *hetR* and *ntcA* genes.

Fig. 3. Primer extension analysis of the expression of *hetR* in *Anabaena* sp. strain PCC 7120, the *ntcA* strain CSE2 and *hetR* strains DR884a and 216. RNA was isolated from cells grown with ammonium (lanes labeled +) or grown with ammonium and deprived of combined nitrogen for 6 h (lanes labeled -). Oligonucleotides used as primers were HR2 (A, B), HR3 (C), and HR7 (D). Similar results were obtained for the -696 and -728 tsps (shown in panels A, B) using oligonucleotide hetRPEX3 (not shown). Sequencing ladders were generated with the corresponding oligonucleotides and plasmid pWB216S2.4 (Buikema and Haselkorn, 1991). The putative tsps are indicated by arrowheads. WT, wild type strain PCC 7120.

Fig. 4. Primer extension analysis of the expression of *ntcA* in *Anabaena* sp. PCC 7120, the *ntcA* strain CSE2 and the *hetR* strain DR884a. (A) RNA was isolated from cells grown with ammonium (lane labeled 0), or grown with ammonium and deprived of combined nitrogen for 6, 12 or 24 hours. (B) RNA was isolated from cells grown with ammonium (lane labeled 0), from cells grown with ammonium and deprived of combined nitrogen for 9 h or from isolated heterocysts (lane labeled H). (C) RNA was isolated from cells grown in the presence of ammonium (NH_4^+), atmospheric nitrogen (N_2) or nitrate (NO_3^-). Oligonucleotides used as primers were NA1 (A, B) and NA7 (C). Sequencing ladders were generated with the corresponding oligonucleotides and *ntcA*-containing plasmid pCSE36 (J. E. Frías, unpublished). The putative tsps are indicated by arrowheads. WT, wild type strain PCC 7120. Note: as a routine control

of the lack of contamination of RNA from heterocysts with RNA from vegetative cells, we carry out hybridizations to *rbcL*, encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase that is expressed only in vegetative cells (Elhai and Wolk, 1990). Hybridization to *nifH*, encoding nitrogenase reductase that is expressed only in the mature heterocyst, is used as a control of the quality of the heterocysts RNA. The RNA preparation from isolated heterocysts used in the experiment shown in panel B did not show hybridization to a probe of the *rbcL* gene, but did show hybridization to a probe of the heterocyst-specific *nifH* gene (not shown).

Fig. 5. Time course of the expression of the *hetC* gene in *Anabaena* sp. strain PCC 7120 and the *hetR* strain DR884a upon nitrogen step-down. Primer extension assays were carried out with RNA isolated from cells grown with ammonium (lanes labeled 0) or grown with ammonium and incubated in combined nitrogen-free medium for 3, 6, 9, 12 or 24 h. The oligonucleotide used as primer was HC2. The sequencing ladder was generated with the corresponding oligonucleotide and plasmid pCSAM83 (Muro-Pastor *et al*., 1999). The tsp located at position -571 is indicated by an arrowhead. WT, wild type strain PCC 7120.

Muro-Pastor et al., FIG 1
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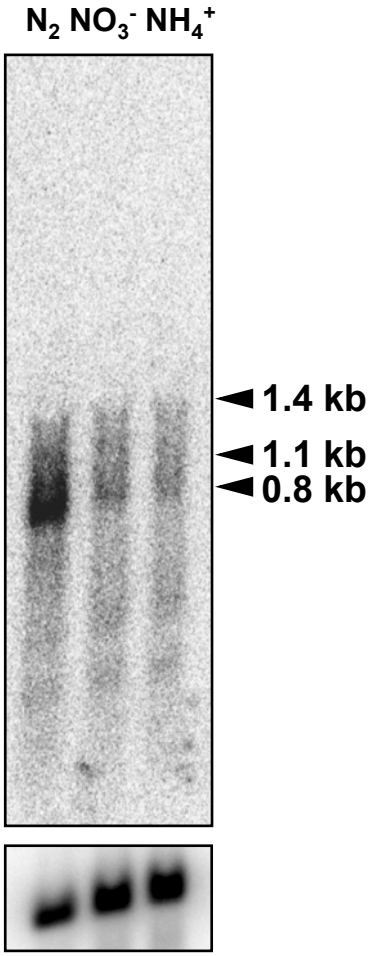

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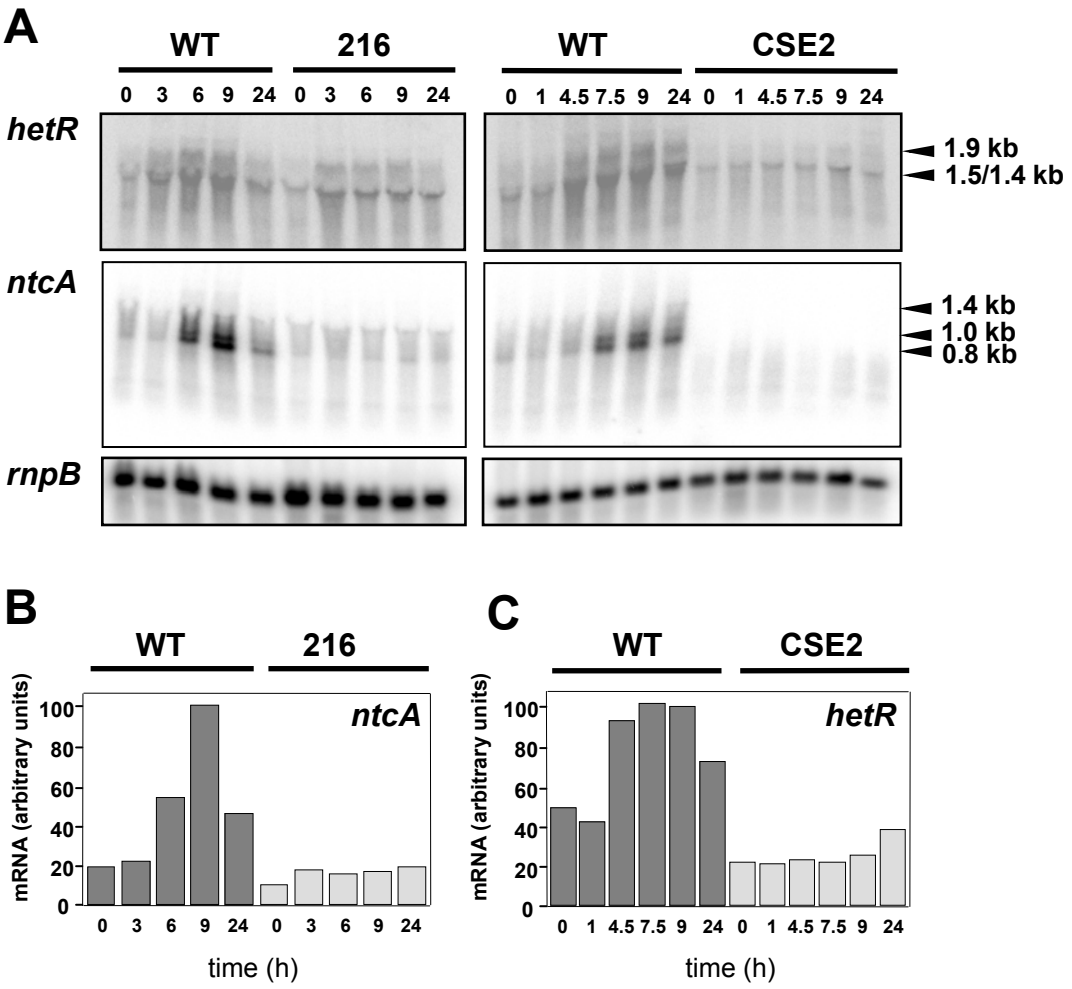

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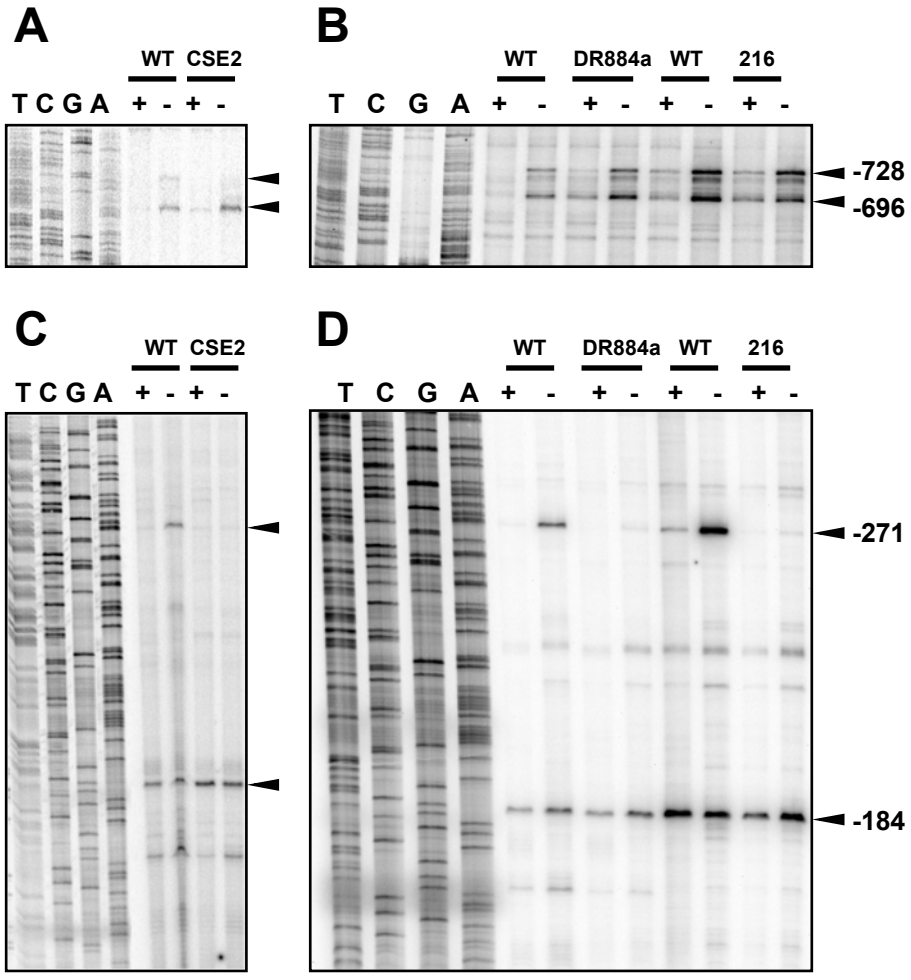

Muro-Pastor et al., FIG 4
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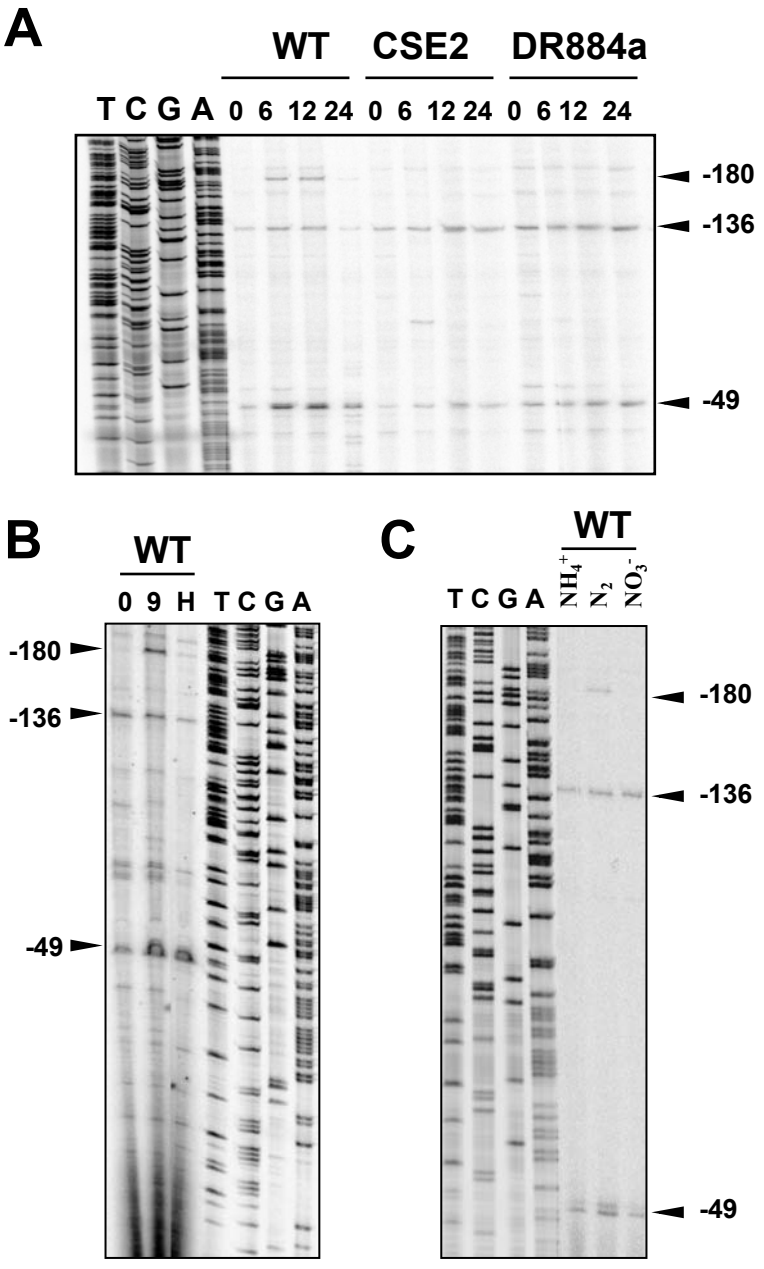

Muro-Pastor et al., FIG 5
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