NtcA is responsible for accumulation of the small isoform of ferredoxin:NADP oxidoreductase

Amin Omairi-Nasser,1† Carla V. Galmozzi,2†§ Amel Latifi,3 M. Isabel Muro-Pastor2 and Ghada Ajlani1

1Institut de Biologie et de Technologie de Saclay, Centre National de la Recherche Scientifique and Commissariat à l’Energie Atomique, 91191 Gif-sur-Yvette, France
2Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC-Universidad de Sevilla, Américo Vespucio 49, 41092 Seville, Spain
3Aix-Marseille Université, Laboratoire de Chimie Bactérienne, Centre National de la Recherche Scientifique, 31 chemin J. Aiguier, 13009 Marseille, France

In several cyanobacteria, petH, the gene encoding ferredoxin:NADP oxidoreductase (FNR), is transcribed from at least two promoters depending on growth conditions. Two transcripts (short and long) are translated from two different translation initiation sites, resulting in two isoforms (large and small, respectively). Here, we show that in Synechocystis PCC6803 the global transcriptional regulator NtcA activates transcription from the distal petH promoter. Modification of the NtcA-binding site prevents NtcA binding to the promoter in vitro and abolishes accumulation of the small isoform of FNR in vivo. We also demonstrate that a similar petH transcription and translation regime occurs in other cyanobacteria. The conditions under which this system operates provide hints for the function of each FNR isoform.

INTRODUCTION

The gene petH encodes ferredoxin:NADP oxidoreductase (FNR), an enzyme that is involved in the last step of oxygenic photosynthesis, in cyanobacteria as well as in chloroplasts. FNR provides NADPH for anabolic reactions and is also implicated in the oxidation of NADPH, produced by catabolism or accumulated due to an imbalance in photosynthetic reactions. Most phycobilisome-containing cyanobacteria possess a large FNR isoform (FNR$_L$) that contains a phycobilisome-linker domain. Fewer strains accumulate a small isoform (FNR$_S$), lacking the linker domain, in addition to FNR$_L$ or as the only FNR form (Thomas et al., 2006).

We showed recently that in Synechocystis sp. PCC6803 (hereafter Synechocystis), following nitrogen starvation, petH produces a transcript bearing a 5′-untranslated region that is longer than the one transcribed under standard conditions. The longer transcript folds into a secondary structure that inhibits FNR$_L$ translation initiation and promotes that of FNR$_S$ (Omairi-Nasser et al., 2011). Although FNR isoform accumulation was not examined in the cyanobacterium Anabaena sp. strain PCC7120 (hereafter Anabaena), its transcript organization is similar to that of Synechocystis. Two transcripts were found for the Anabaena petH; the shorter is constitutive, whilst the longer is NtcA-dependent and mainly located in heterocysts (Valladares et al., 1999).

NtcA is a global transcriptional regulator that belongs to the cAMP receptor protein (Crp)/Fnr bacterial superfamily, and regulates nitrogen and carbon assimilation genes in cyanobacteria in response to 2-oxoglutarate. 2-Oxoglutarate is an intermediate of the TCA cycle that provides the carbon skeleton for nitrogen incorporation into amino acids. In cyanobacteria, 2-oxoglutarate also functions as a regulatory effector for NtcA and PII, whose activities alter gene expression and metabolism (Muro-Pastor et al., 2001; Körner et al., 2003; Osanai et al., 2006; Luque & Forchhammer, 2008). In many cases, NtcA-activated promoters are similar to class II Crp-dependent promoters, in which the transcription factor binds to a consensus sequence centred at ~40 bases upstream from the transcription start point (Luque & Forchhammer, 2008). A consensus sequence for NtcA binding has been defined as GTAN$_5$TAC (Herrero et al., 2001; Mitschke et al., 2011b). The recently solved NtcA structure shows that NtcA is a dimeric protein with a very similar overall structure to that of Crp (Llácer et al., 2010; Zhao et al., 2010).

†These authors contributed equally to this work.
‡Present address: Department of Molecular Genetics & Cell Biology, University of Chicago, Chicago, IL 60637, USA.
§Present address: Zentrum für Molekulare Biologie der Universität Heidelberg, Heidelberg, Germany.

Abbreviations: Crp, cAMP receptor protein; EMSA, electrophoretic mobility shift assay; FNR, ferredoxin:NADP oxidoreductase; L, large; S, small.
Putative NtcA-binding sites are present upstream from the promoter that produces the longer petH transcript in *Synechocystis*. We identified the actual binding site, centred at −41.5 bases from the distal petH transcription start point, and showed that NtcA is required for FNR<sub>S</sub> synthesis in *Synechocystis*. Moreover, the petH transcription and translation mechanisms, leading to the accumulation of two FNR isoforms, are conserved in *Anabaena* and in *Synechococcus* sp. PCC7002.

An NtcA-binding site was identified upstream from the transcription start point producing a longer petH transcript in *Anabaena* (Valladares et al., 1999). Here, we show that NtcA binding to this distal promoter is responsible for FNR<sub>S</sub> translation in heterocysts.

**METHODS**

**Strains and growth conditions.** WT and mutants of *Synechocystis* were grown photoautotrophically, at 33 °C, in a CO<sub>2</sub>-enriched atmosphere and under continuous light (50 μE m<sup>−2</sup> s<sup>−1</sup>), which is standard growth conditions in our laboratory. The medium composition is described in Ughy & Ajlani (2004). For nitrogen starvation, cells were harvested by centrifugation and resuspended in a medium where NaCl replaced NaNO<sub>3</sub>. When appropriate, media were supplemented with 5 μg streptomycin ml<sup>−1</sup> and 50 μg spectinomycin ml<sup>−1</sup> or 50 μg kanamycin ml<sup>−1</sup>. *Synechococcus* sp. PCC7002 was grown under the same conditions used for *Synechocystis* except that A<sub>S</sub> medium was used (Stevens & Porter, 1980). *Anabaena* sp. PCC7120 WT and CSE2 strains were grown photoautotrophically, at 30 °C, in BG11C, which is BG11 medium without NaNO<sub>3</sub> and with 10 mM NH<sub>4</sub>Cl supplemented with 6 mM NH<sub>4</sub>Cl plus 12 mM TE5 (pH 7.5) and bubbled with 1% CO<sub>2</sub> in air until the exponential phase. Then the ammonium was eliminated and the cells incubated for the time indicated in BG11C (nitrogen free), with 1% CO<sub>2</sub> in air under continuous light (500 μE m<sup>−2</sup> s<sup>−1</sup> Em atmosphere and under continuous light (500 μE m<sup>−2</sup> s<sup>−1</sup>). When appropriate, media were supplemented with 5 μg streptomycin ml<sup>−1</sup> and 50 μg spectinomycin ml<sup>−1</sup> or 50 μg kanamycin ml<sup>−1</sup>. *Synechocystis* NtcA–His<sub>6</sub>, expressed and purified as described above, was used in gel retardation assays. The P<sub>petH</sub> promoter probes were obtained by *Spe*I/*Sal*I digestion of plasmids carrying WT or modified versions of the NtcA-binding site. DNA fragments were end-labeleld with [α-<sup>32</sup>P]dCTP using Sequenase version 2.0 enzyme. The binding reactions and electrophoresis were carried out as described previously (Muro-Pastor et al., 1996).

**Mutagenesis and plasmid construction.** Mutagenic PCRs were performed on a plasmid carrying a 232 bp *Spe*I/*Sal*I fragment, containing the petH 5′-non-coding region from *Synechocystis*, using mutagenic primer pairs 5′-CTAGCATGATGATCCTCCATACGG-3′/5′-GGTCGTATACTGTTGAATC-3′ for the X mutation and 5′-CCTTTAGATCGTTGTTAGG-3′/5′-GTAACCGTATGGTAC-3′ for the P mutation. The resulting plasmids were sequenced to verify the fidelity of the PCR amplification. To create the cargo plasmids, the modified *Spe*I/*Sal*I DNA fragment, which spans positions −5 to +144 with respect to the distal petH transcription start point, was cloned into the PET24a (+) plasmid (Novagen) to generate pSNtcA. Exponentially growing *Escherichia coli* BL21 cells transformed with pSNtcA were treated with 0.5 mM IPTG for 4 h. The C-terminal His-tagged version of *Synechocystis* NtcA was purified by nickel-affinity chromatography using His-Bind matrix (Novagen) following the manufacturer’s instructions. For further purification, the sample was subjected to gel filtration chromatography using a HiLoad 16/60 Superdex 75 column (GE Healthcare) running on an Akta FPLC system.

**RESULTS**

NtcA binding to WT and modified versions of the *Synechocystis* petH promoter

To test whether NtcA binds to the *Synechocystis* petH promoter, electrophoretic mobility shift assays (EMSAs) using purified *Synechocystis* NtcA protein were performed. The protein was expressed in *E. coli* and purified as a His-tagged variant. Binding assays were performed using an *Spe*I/*Sal*I DNA fragment, which spans positions −85 to +144 with respect to the distal petH transcription start point. It has been shown that >8 nt might separate the NtcA-binding triplets (Jiang et al., 2000), so two putative binding sites (GAT<sub>N</sub>N<sub>T</sub>AAC and GTG<sub>T</sub>N<sub>T</sub>A, centred at −41.5 and −42.5 upstream from the distal petH transcription start point, were considered. We tested fragments containing modified versions of both putative sites; X, creating an *Xba*I restriction site, and P, creating a *Pst*I site where only the putative site N<sub>T</sub> was modified (Fig. 1).

When purified NtcA was incubated with the WT-labelled probe, an NtcA–DNA complex was clearly detected (Fig. 1a); however, when the X probe was used, no NtcA–DNA complex was detected (Fig. 1b). The mutations present in the P probe did not prevent NtcA binding *in vitro* (Fig. 1c). These results indicate that NtcA binds *in vivo* to the GAT<sub>N</sub>N<sub>T</sub>AAC site, which is centred at −41.5 upstream from the distal petH transcription start point.

NtcA binding is required for FNR<sub>S</sub> accumulation in *Synechocystis*

To determine whether the identified NtcA-binding site operated *in vivo*, the X mutation linked to the Ω cassette
was introduced in *Synechocystis*, yielding mutant NBX (Fig. 2a). Total segregation of the chromosomes was confirmed by PCR and restriction analysis, in which the presence of the *Xba*I site confirmed the integration of the modified NtcA-binding site into the segregated chromosome (Fig. 2b).

FNR isoform accumulation upon nitrogen starvation was examined in total-protein extracts from NBX, which carried the X mutation plus an *O* cassette insertion 115 bases upstream from the distal transcription start point, the WT and BX, a strain carrying a WT *petH* allele plus the omega cassette at the same site as NBX. Fig. 2(c) shows that, unlike the WT and BX, NBX did not accumulate FNRS even after 3 days of nitrogen starvation. A similar experiment performed with the P mutation showed a WT behaviour for FNR isoform accumulation, which excludes NtcA binding to the putative N10 site in vivo (data not shown).

We have shown previously that FNRS accumulation depends on the *petH* distal promoter, producing a long transcript whose translation yields FNRS (Omairi-Nasser et al., 2011). The absence of FNRS in a strain lacking the NtcA-binding site implies that NtcA is required for *petH* transcription from the distal promoter. These results confirmed the requirement of the longer transcript for the synthesis of FNRS and established its transcription activation by NtcA.

**FNRS accumulation in *Synechococcus* sp. PCC7002**

Recent transcriptome studies in *Synechococcus* sp. PCC7002 showed that *petH* transcription proceeds from a proximal transcription start point, located 87 bases upstream from the ORF, under standard conditions, and from a distal transcription start point, located 283 bases upstream from the ORF, operating under nitrogen starvation (Ludwig & Bryant, 2012). We performed Western blots on total extracts from *Synechococcus* sp. PCC7002 grown under standard and nitrogen starvation conditions. Fig. 3 shows that FNRS was expressed upon nitrogen starvation, consistent with the transcript results.

A putative NtcA-binding site was found upstream from the distal *petH* promoter in *Synechococcus* sp. PCC7002 (Fig. 6a, b). Thus, the situation in this cyanobacterium turns out to be similar to the situation described for *Synechocystis*, where FNRS accumulation follows an NtcA-induced and longer *petH* transcript.

**FNRS accumulation in *Anabaena* PCC7120**

In *Anabaena* PCC7120, *petH* is transcribed from a constitutive transcription start point, located 63 bases
upstream from the ORF, and from an NtcA-regulated transcription start point, located 188 bases upstream from the ORF (Valladares et al., 1999). To test whether the NtcA-regulated long transcript is translated into a small FNR isoform, we performed Western blots on total extracts from the WT as well as from an ntcA insertional mutant (CSE2; Frías et al., 1994) grown with ammonia or in the absence of combined nitrogen. Fig. 4 shows that FNR_S accumulates upon nitrogen step-down in WT cells, whilst it failed to accumulate in the ntcA mutant. A small amount of FNR_S was detected in the presence of nitrogen in the WT (7120WT, 0), but also when NtcA was absent (NtcA –, all wells).

As the distal promoter was shown to be the main promoter operative in the heterocysts (Valladares et al., 1999), purified heterocysts were tested for the presence of FNR_S. Fig. 5(b) shows clearly that FNR_S is the major isoform in purified heterocysts. Heterocyst extract purity was estimated by evaluating their relative content of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RbcL, which is expressed only in vegetative cells). As shown in Fig. 5, isolated heterocysts had similar amounts of RbcL and FNR_L, which suggested that mature heterocysts contain almost exclusively FNR_S.

**DISCUSSION**

This work shows that petH regulation is similar in cyanobacteria capable of FNR_S synthesis. An ORF-proximal transcription start point produces a transcript that is translated into FNR_L, whilst a distal transcription start point controlled by NtcA, produces a longer transcript that is translated into FNR_S.

When *Synechocystis* is grown under standard conditions, FNR_L is the major isoform but traces of FNR_S are present. The distal petH transcription start point was detected in addition to the proximal transcription start point under standard conditions in the genome map of *Synechocystis* transcription start points (Mitschke et al., 2011a). Here, we show that NtcA binding to the distal promoter is required for FNR_S synthesis that depends on the distal petH promoter activity (Omairi-Nasser et al., 2011). NtcA was shown to be essential for *Synechocystis* under all known conditions (García-Domínguez et al., 2000), which implies that NtcA is present even when nitrogen is available. Therefore, the slight accumulation of FNR_S and the presence of the longer transcript in the WT *Synechocystis* are due to the presence of NtcA under standard conditions.

A slight difference was found in *Synechococcus* sp. PCC7002. Whilst FNR_S accumulates under nitrogen starvation, it was not detected under standard conditions. In a recent transcriptome work, the long petH transcript, in addition to
the short transcript, was detected under standard conditions (Ludwig & Bryant, 2012; M. Ludwig & D. A. Bryant, personal communication). The absence of FNRS under the standard conditions used in our work, might either result from a poor recognition of the *Synechococcus* sp. PCC7002 FNRS, by the *Synechocystis* antibody, or from the different growth conditions used in each laboratory.

The low level of FNRS detected in *Anabaena* grown in the presence of ammonia, as well as in the NtcA-deficient mutant (Fig. 4), suggests that NtcA does not tightly control transcription from the distal promoter in this cyanobacterium. It is noteworthy that a putative −35 element overlaps the NtcA-binding site in *Anabaena* (Fig. 6a, c); this might be responsible for the low level of FNRS detected in the absence of NtcA. It was shown that NtcA was responsible for the upregulation of the distal petH promoter in *Anabaena* (Valladares et al., 1999); here, we showed that NtcA is required for the accumulation of a higher level of FNRS.

In addition to its photoautotrophic growth ability, *Anabaena* can fix molecular nitrogen when combined with heterocysts. Heterocysts have been shown to contain 14 times more FNR than vegetative cells (Razquin et al., 1996). Since ~10% of the cells in a nitrogen-fixing culture are heterocysts, the equal amounts of FNRS and FNR_L in a nitrogen-fixing culture are heterocysts, the equal amounts of FNRS and FNR_L in a nitrogen-fixing culture are heterocysts. In another experiment, we showed that purified heterocysts contained almost exclusively FNRS, whilst FNR_L is the major isofrom in ammonia-grown vegetative cells. This suggests that FNRS function is related to photosynthesis and sugar catabolism, whilst FNR_L functions in an environment where sugar catabolism is activated to sustain nitrogen fixation.

Obligate photoautotrophs like *Synechococcus elongatus* and *Thermosynechococcus elongatus* do not contain a second initiating codon and do not accumulate FNRS (Thomas et al., 2006). In a recent transcriptome study of *S. elongatus*, the petH transcription start point was located 135 bases upstream from the ORF under standard conditions (Vijayan et al., 2011). Unfortunately, no transcription data are available for this cyanobacterium under nitrogen-limited conditions. However, the fact that strains capable of FNRS synthesis such as *Synechocystis* sp. PCC6803, *Synechococcus* sp. PCC7002 and *Anabaena* sp. PCC7120 are facultative heterotrophs strongly suggests that FNRS function is associated with catabolism.

**Potential interaction of NtcA with its binding site**

Comparison of the petH binding sites of different species indicates that the first nucleotide position of the consensus triplet. As in the Crp structure, the F-helix of NtcA ensures DNA recognition. The F-helix of Crp was shown to exhibit sequence preferences at specific positions (GTGA) within each DNA half-site, which are mediated by Arg180, Glu181 and Arg185 (Lawson et al., 2004). In the Crp–DNA complex, Arg180 interacts with guanine (O6) and N7 atoms) of the GC pair at position 1 of its consensus; a similar interaction may occur in the NtcA–DNA complex through Arg187 (or 186, depending on amino acid numbering) (position 1, Fig. 6b). In place of Glu181 of Crp, a Val is found in NtcA, which is believed to accommodate an AT (instead of a GC) pair at position 3 and explains the GTA triplet in the NtcA-binding consensus (Llacer et al., 2010). However, Arg185 in the Crp–DNA complex interacts with guanine (O6 and/or N7) of GC at position 3, but also with thymine (O4) of the AT pair at position 4 (Lawson et al., 2004). In the NtcA complex, a similar interaction could occur between Arg192
or 191) and the adenine ($N^2$) of the AT pair at position 3 (Fig. 6b). In this context, NtcA binding would be strengthened by the presence of an AT pair at position 4, as Arg192 would also interact with the thymidine ($O^4$).

In *Synechocystis*, the position 3 AT pair is not found in the 5’ half of the petH NtcA-binding site, but an AT pair is present in both half-sites at position 4 (Fig. 6a, a). In *Synechococcus* PCC7002, the position 3 AT pair is not conserved in the 3’ half, but again both half-sites retained an AT pair at position 4 (Fig. 6a, b). In *Anabaena*, none of the position-3 AT pairs are conserved and only the one at position 4 of the 3’ half is conserved (Fig. 6a, c).

Therefore, the first nucleotide after the GTA consensus triplet could play a role in NtcA binding. Further experiments are required to test this proposal.

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