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Excision of the 59-kb *fdxN* DNA element is required for transcription of the *nifD* gene in *Anabaena* PCC 7120 Heterocysts

Running title: Expression of *nif* genes in *Anabaena*

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ABSTRACT

In the heterocyst-forming cyanobacterium *Anabaena* (also *Nostoc*) sp. strain PCC 7120, the nitrogen-fixation (*nif*) genes are expressed specifically in heterocysts during the late stages of development. We used *gfp*-reporter fusions to examine the regulation of the *nifH* and *nifD* genes. Plasmid-borne reporter fusions containing up to 500-bp of the *nifH* upstream region, which extended into the *nifU* gene, did not show developmentally regulated GFP fluorescence after removal of a nitrogen source from the growth medium, which indicated that sequences essential for transcriptional regulation are outside of the tested regions. Therefore, a *gfp*-reporter fusion was engineered into the chromosome at the 5' end of *nifD* to produce strain AMC1774. This reporter construct at the native locus showed strong developmentally regulated GFP expression in differentiating heterocysts by 18 hours after removal of combined nitrogen. We then screened for UV-induced mutants of AMC1774 that differentiated heterocysts but failed to show GFP-reporter fluorescence. Several dark mutants were obtained and then complemented with an expression library. One mutant was complemented with a clone that contained the *xisF* and *alr1460* genes. The *xisF* gene is required for excision of a 59,428-bp DNA element present within the *fdxN* gene in the *nifB-fdxN-nifS-nifU* gene cluster, which is upstream of the *nifHDK* gene cluster. Analysis of the original UV-induced mutant showed that it was defective for excision of the *fdxN* DNA element. Together, our data shows that transcription of the *nifHDK* genes requires a distant promoter upstream of the *fdxN* DNA element. We used a *gfp* transcriptional reporter to demonstrate developmentally regulated promoter activity from the intergenic

region upstream of *nifB*. We also show that the promoter for a second *nifH* gene, *nifH2* (*alr0874*), lies proximal to *nifH2* in its upstream intergenic region.

INTRODUCTION

The filamentous cyanobacterium *Anabaena* (also *Nostoc*) sp. strain PCC 7120 (hereafter *Anabaena* PCC 7120) responds to nitrogen deprivation by differentiating a developmental pattern of single heterocysts separated by about 10 to 20 photosynthetic vegetative cells. Heterocyst structure and metabolic activity serve to spatially separate the oxygen-sensitive process of nitrogen fixation from oxygen-producing photosynthesis in vegetative cells. *Anabaena* PCC 7120 contains a single continuous cluster of 17 genes that encode nitrogenase and its cofactors. The *nifHDK* gene cluster encodes the major structural proteins of the molybdenum-containing nitrogenase enzyme complex: *nifH* encodes dinitrogenase reductase and *nifD* and *nifK* encode the dinitrogenase alpha and beta subunits, respectively. Previously, the organization of the *nifHDK* genes and northern RNA blot data were consistent with these genes forming an operon (Haselkorn 1986; Golden et al. 1991). Upstream of the *nifHDK* gene cluster is another set of *nif* genes, *nifB-fdxN-nifS-nifU*, that are expressed at low levels (Fig. 1A) (Mulligan and Haselkorn 1989). The *nifB*, *nifS*, and *nifU* genes are involved in the synthesis of the dinitrogenase FeMo-cofactor and *fdxN* encodes a bacterial-type ferredoxin (Rubio and Ludden 2008).

Although the *Anabaena* PCC 7120 *nifH* gene is expressed at high levels in heterocysts and it was the first nitrogen fixation gene to be sequenced (Mevarech et al. 1980), progress on understanding the developmentally regulated expression of the *nif* genes has been slow. Heterocyst-specific *nif* genes are developmentally regulated and expressed specifically in heterocysts between 18 and 24 hours after nitrogen deprivation (Golden et al. 1991; Wolk 1996; Flaherty et al. 2011; Mella-Herrera et al.

2011), when the heterocyst environment becomes microoxic, thus protecting the nitrogenase enzyme from irreversible inactivation by oxygen. For *Anabaena* PCC 7120, heterocyst-specific mRNA 5' ends upstream of the *nifH*, *nifB*, and *fdxH* genes were mapped but the methods employed in this early work did not demonstrate promoter activity for these putative transcription start sites (TSSs) (Haselkorn et al. 1983; Bohme and Haselkorn 1988; Mulligan and Haselkorn 1989). Later, differential RNA-seq was used to provide a genome-wide map of TSSs for genes expressed at early times during differentiation (Mitschke et al. 2011). This work defined a DIF(+) sequence motif near the -35 position of many genes that is associated with early heterocyst-specific expression, however, the experimental design did not identify TSSs for *nif* genes, which are expressed at the late stages of differentiation.

Recent progress in understanding the regulation of *nif*-gene expression in the related strain *Anabaena variabilis* can now be used as a paradigm for other cyanobacterial strains (Ungerer et al. 2010; Pratte et al. 2013; Pratte and Thiel 2014; Thiel and Pratte 2014; Pratte et al. 2015; Pratte and Thiel 2016; Vernon et al. 2017). In *A. variabilis*, two promoters contribute to the expression of the *nifHDK1* genes: one promoter upstream of *nifB1* contributes to the majority of the transcripts and a second weak promoter is within *nifU1* (Ungerer et al. 2010). As in *Anabaena* PCC 7120, *A. variabilis* has much higher levels of *nifHDK* transcripts compared to transcripts for the upstream *nifB-fdxN-nifSU* genes. In *A. variabilis*, stem-loop structures are involved in the differential stability of the *nifH1, fdxH1*, and downstream transcripts (Ungerer et al. 2010; Pratte and Thiel 2014; Pratte et al. 2015). Pratte and Thiel analyzed an *xisA* mutant that cannot excise the 11-kb *nifD* DNA element and concluded that the *nifB1*

93 promoter contributed to the expression of genes downstream of the *nifD* DNA element,
94 but many of the downstream genes still showed low to moderate levels of transcripts
95 correlated with processing at the stem-loop sites (Pratte and Thiel 2014).
96 In addition, transcription factors involved in regulation of the *nif* genes have been
97 identified in *A. variabilis*. Two homologous transcription factors were shown to regulate
98 the *nif1* and *nif2* gene clusters (Pratte and Thiel 2016). CnfR1 activates heterocyst-
99 specific expression of the *nifB1* promoter, and CnfR2 activates the *nifB2* promoter in
100 vegetative cells under anaerobic conditions.
101 In *Anabaena* PCC 7120, three developmentally programmed site-specific DNA
102 rearrangements result in the excision of three DNA elements from within the open
103 reading frame of genes on the chromosome during the late stages of heterocyst
104 differentiation (Golden and Yoon 1998). Two of the DNA elements are located within the
105 nitrogen fixation gene cluster and a third element is in the *hupL* gene. For the two
106 elements in the *nif* gene cluster, one rearrangement excises an 11,278 bp DNA element
107 from within the *nifD* gene and is catalyzed by the *xisA* gene product located on the
108 element (Golden et al. 1985; Golden and Wiest 1988). The second rearrangement
109 excises a 59,428-bp DNA element from within the *fdxN* (bacterial-type ferredoxin) gene
110 (Golden et al. 1988). The *xisF* gene, located on the left end of the *fdxN* element,
111 encodes a site-specific recombinase that is required for excision of the *fdxN* element in
112 heterocysts and is sufficient to cause rearrangement of a DNA substrate molecule in *E.*
113 *coli* (Carrasco et al. 1994). Developmentally regulated excision of the fdxN DNA
114 element in heterocysts requires *xisF* and the downstream *xisH* and *xisI* genes
115 (Ramaswamy et al. 1997). Mutants that fail to excise either DNA element from the

heterocyst chromosome are defective for diazotrophic growth on media lacking a source of combined nitrogen. Although the regulated excision of the three DNA elements from the genes in which they reside is essential for those genes to function in heterocysts, the origin of the DNA elements and any potential selective advantage they may provide remains unclear.

In the current study, we used *gfp* reporter fusions to locate cis-acting promoter sequences required for transcription of the *nifHDK* genes in *Anabaena* PCC 7120. We show that excision of the *fdxN* element present in the *nifB-fdxN-nifSU* gene cluster is required for the transcription of the downstream *nifHDK* genes, which indicates that transcription of these genes initiates upstream of the *fdxN* element. We show with *gfp* transcriptional reporter strains that the *nifB* upstream intergenic region contains a developmentally regulated promoter. Taken together our results indicate that transcription of the downstream *nifHDK* genes originates from the far upstream *nifB* promoter. Finally, *gfp*-reporter transcriptional fusions were used to demonstrate that another developmentally regulated *nif* promoter is present in the upstream intergenic region of the *nifH2* (*alr0874*) gene.

MATERIALS AND METHODS

Strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *Anabaena* PCC 7120 and its derivatives were grown in BG-11 or BG-11_0 (which lacks sodium nitrate) medium at 30°C as previously described (Golden et al. 1991). Cultures were grown under fluorescent white light illumination at 75-100 µmol photons m^{-2} s^{-1} measured with a Biospherical Instruments QSL-100 light meter.

Plasmids were transferred to *Anabaena* PCC 7120 by conjugation from *Escherichia coli* following published protocols (Elhai et al. 1997; Khudyakov and Golden 2001; Liu and Golden 2002). For *Anabaena* PCC 7120 strains carrying plasmids, the antibiotics neomycin (Nm; 25 μg/ml) and streptomycin (Sm; 2 μg/ml) plus spectinomycin (Sp; 2 μg/ml) used together were used for selection on BG-11 and BG-11_0 agar medium in plates; the antibiotic concentrations were reduced by half for BG-11 and BG-11_0 liquid cultures. Heterocyst differentiation was induced by nitrogen deprivation as previously described (Aldea et al. 2007).

E. coli strains were maintained in LB (Lennox L) liquid or agar-solidified medium supplemented with appropriate antibiotics at standard concentrations (Elhai et al. 1997; Khudyakov and Golden 2001; Liu and Golden 2002).

Plasmid constructions. Plasmids are listed in Table 1 and primers are listed in Table 2. Reporter plasmids were constructed in the shuttle vector pAM1956 (Yoon and Golden 1998). Reporter plasmids pAMKK5, pAMKK6, and pAMKK25 were constructed by amplifying different *nifH* upstream regions using primers KKO-9 and KKO-10 (221-bp fragment), KKO-11 and KKO-12 (350-bp fragment), and KKO-15 and KKO-16 (500-bp fragment), respectively. Amplified fragments were cloned into KpnI (for the 221-bp fragment) or SalI and SacI sites of pAM1956. The suicide *gfp* reporter vector pAMKK1 was constructed by amplifying *gfpmut2* from pAM1956 using primers KKO-1 and KKO-2 and cloning the fragment into SpeI and SacI restriction sites of the conjugal suicide vector pRL277 (Elhai et al. 1997). The *nifD-gfp* reporter suicide plasmid pAMKK2 was designed to integrate upstream of the native *nifD* gene and was constructed by cloning a PCR-amplified fragment, using primers KKO-3 and KKO-4, of the *nifD* upstream

region from +3-bp to -2271-bp relative to the *nifD* ATG into XhoI and SpeI sites of pAMKK1. To test the *nifD* upstream region in pAMKK2 for regulated promoter activity at a distant neutral site, the suicide plasmid pAMKK3 was designed to integrate into the gas vesicle protein (*gvp*) gene locus. A 1.5-kb *gvp* gene region was amplified using primers KKO-5 and KKO-6 and cloned into SacI and PstI sites on the P_{nifD}-*gfp* plasmid pAMKK2. The P_{nifB}-*gfp* transcriptional reporter shuttle plasmid pAMKK4 was constructed by amplifying the *nifB* upstream region with primers KKO-7 and KKO-8, containing SacI and Acc651 restriction sites, respectively, and cloning the amplified fragment into pAM1956. The P_{nifH2}-*gfp* transcriptional reporter pAMKK26 was constructed by amplifying the *nifH2* upstream region with primers KKO-9 and KKO-10, containing SacI and Acc651 restriction sites, respectively, and cloning the amplified fragment into pAM1956. All plasmid constructs were verified by DNA sequencing.

Isolation of mutants by UV mutagenesis. UV mutagenesis of the AMC1774 reporter strain was performed according to published protocols (Wolk et al. 1988) with minor modifications. Following UV exposure, culture flasks were wrapped in yellow cellophane and were grown under fluorescent white light. Cultures were sonicated to fragment the filaments before being plated on BG-11_0 plates containing streptomycin and spectinomycin to obtain individual colonies. All resulting colonies were patched to BG-11 and BG-11_0 plates, and the BG-11_0 plates were screened by fluorescence microscopy for Het+ clones that had lost *nifD-gfp* reporter fluorescence.

Complementation with an expression library. A conjugal expression library was used for genetic complementation and gene overexpression experiments (Liu and Golden 2002). Pooled library plasmid DNA was transformed into competent *E. coli*

strain AM1358 and then transferred into the dark UV mutant of strain AMC1774 by triparental conjugation with *E. coli* strain AM1460 as previously described (Liu and Golden 2002). Conjugation mixtures were plated on Millipore HATF filters laid on BG-11_0 plates containing 5% LB medium and incubated at 30°C under 75-100 µmol photons m^{-2} s^{-1}. After 24 hours, the filters were moved onto BG-11_0 plates containing streptomycin and spectinomycin (2 µg/ml each) to select for the reporter construct in AMC1774 and neomycin (25 µg/ml) to select for the library plasmids. Approximately 30,000 exconjugant colonies were screened for diazotrophic growth followed by screening the Het+, yellow Nif- colonies for changes in GFP-reporter fluorescence.

Screen of the expression library in AMC1774. The conjugal expression library was screened in the AMC1774 reporter strain to obtain dark strains using methods that were essentially the same as the complementation experiments described above. 43,500 colonies were screened for colonies that were defective for diazotrophic growth, and that were Het+ and failed to express the *nifD-gfp* reporter.

Microscopy. Fluorescence and bright-field images were captured using an Olympus IX70 inverted microscope with a Hamamatsu OrcaER C4742-95 charge-coupled-device camera and Simple PCI software version 6.1 as previously described (Aldea et al. 2007). A Piston green fluorescent protein (GFP) band-pass filter set (no. 41025; Chroma Technology Corp.) was used for fluorescence images. Photomicrographs were minimally processed with Adobe Photoshop 8.0 to improve brightness and contrast.

RESULTS

Sequences in the *nifH* upstream intergenic region do not drive transcription of a *nifH-gfp reporter*. To identify sequences upstream of the *nifH* gene in *Anabaena* PCC 7120 that are required for transcription of the *nifHDK* gene cluster, we initially constructed several transcriptional *gfp*-reporter fusions with sequences upstream of *nifH* on shuttle plasmid pAM1956 (Fig. 1A) (Yoon and Golden 1998). We first constructed strain KKC5 containing the intergenic region (221-bp) between *nifU* and *nifH* fused to *gfp* on pAM1956. Because a putative *nifH* TSS had been mapped to position -123 relative to the *nifH* start codon (Haselkorn et al. 1983), we expected the *nifHDK* promoter to be in this region. However, KKC5 did not show regulated GFP expression in heterocysts after being transferred to medium without a source of combined nitrogen. Strains KKC6 and KKC25, which contain 350-bp and 500-bp fragments, respectively, of the *nifH* upstream region fused to *gfp* on pAM1956 also did not show GFP expression in heterocysts after nitrogen deprivation (Fig. 1A and data not shown). It should be noted that the requirement for molecular oxygen for GFP maturation and fluorescence does not affect its use as a reporter during heterocyst development and in newly formed heterocysts (Yoon and Golden 1998, 2001; Mella-Herrera et al. 2011; Videau et al. 2014). To determine if sequences within the *nifH* ORF might influence stability of the *nifH* transcripts and affect reporter expression, we made reporter fusions of *gfp* fused to fragments that started in the 3' end of the upstream *nifS* gene and extended to the *nifH* ATG and to positions 54-bp, 99-bp, 250bp, 450-bp, and 750-bp downstream of the *nifH* start codon. No GFP was detected for any of these reporter strains (data not shown).

To confirm the absence of GFP protein in KKC5, KKC6, and KKC25, a western immunoblot experiment was performed that included a positive GFP control. No GFP protein was detected for the three reporter strains (data not shown) supporting the conclusion that there was no *nif*-promoter activity from these *nifH* upstream regions. These results indicate that the promoter necessary for transcription of the *nifHDK* genes is not present within the 500-bp region upstream of *nifH*.

A *nifD-gfp* reporter at the native site in the chromosome is expressed in heterocysts. To mitigate potential problems that could be caused by reporter fusions carried on plasmids, and the possibility that *nif* promoters require a chromosomal context for developmental regulation, we constructed a *gfp* reporter fusion to *nifD* at the native locus in the chromosome. Suicide plasmid pAMKK2 was constructed such that a 2274-bp DNA fragment upstream of *nifD* was fused to *gfp* at the start codon of the *nifD* open reading frame (ORF). The plasmid pAMKK2 was integrated into the chromosome by homologous recombination at the *nif* locus by a single crossover event to produce strain AMC1774 (Fig. 1A), in which the native chromosome region upstream of *nifD* is driving the *gfp* reporter gene. In an initial report, strain AMC1774 was used as a *nif*-gene reporter in the study of the *Anabaena* PCC 7120 *sigE* gene (Mella-Herrera et al. 2011). In cultures grown without nitrate, strain AMC1774 showed strong heterocyst-specific GFP fluorescence (Fig. 1B). Strain AMC1774 is capable of diazotrophic growth, so transcription of the *nif* genes downstream of the suicide plasmid insertion site must be driven by read-through from the upstream promoter or from sequences on the vector. Time-lapse microscopy showed that GFP fluorescence was upregulated by 18

hours after nitrogen deprivation (Mella-Herrera et al. 2011), indicating that the reporter strain was showing normal spatial and temporal regulation.

Isolation of mutants that fail to express the *nifD-gfp* reporter in AMC1774. To identify factors involved in the regulation of the nitrogen fixation genes, UV mutagenesis was performed on AMC1774, and the mutagenized population was screened for mutants that showed abnormal expression of the *nifD-gfp* reporter. We screened approximately 30,000 colonies for mutants that formed heterocysts but did not show developmentally regulated GFP expression. One mutant, KKC3, had normal growth and morphology on BG-11 medium. On BG-11_0 medium, which lacks a source of combined nitrogen, strain KKC3 produced vegetative cells and heterocysts with normal morphology, but did not show GFP reporter fluorescence from heterocysts (Fig. 2A), was unable to continue growth, and had yellowish colonies typical of chlorosis caused by nitrogen starvation.

Complementation of KKC3 with an expression library (Liu and Golden 2002) identified one clone that restored the WT phenotype. The complementing plasmid carried genes *xisF* (*alr1459*) and its flanking gene *alr1460*. Previous studies showed that *xisF* encodes a site-specific recombinase that is required for excision of the *fdxN* element, and that the nearby genes *xisH* (*alr1461*) and *xisI* (*alr1462*) are involved in the developmental regulation of the excision in heterocysts (Carrasco et al. 1994; Ramaswamy et al. 1997).

In addition to screening UV-induced mutants, we also screened for abnormal expression of the *nifD-gfp* reporter after transferring the plasmid expression library (Liu and Golden 2002) of random gene fragments into AMC1774. Altered phenotypes could

result from misregulation of gene expression, the overexpression of intact genes, or expression of partial genes such that the products could interfere with the activity or function of the normal gene product. We screened approximately 43,500 colonies on plates and isolated two dark strains that were unable to grow under conditions of nitrogen deprivation and did not express the *nifD-gfp* reporter. These two strains appeared normal on BG-11 medium and formed heterocysts on BG-11_0 medium, but similar to strain KKC3, had yellowish colonies and were unable to continue growth. One strain, KKC4, contained an expression library plasmid, named 1824-1, that carried ORFs *alr1459* (*xisF*) and *alr1460* (Fig. 2B), and the other strain contained a library plasmid that carried ORFs *alr1459* (*xisF*), *alr1460*, *alr1461* (*xisH*), *alr1462* (*xisI*), and *all1463* (data not shown).

To determine if *alr1460*, a gene of unknown function, is involved in excision of the *fdxN* element, we attempted to subclone the *alr1460* ORF alone into the expression shuttle vector pAM2770 but were unsuccessful for unknown reasons.

Characterization of KKC3 and KKC4 by PCR analysis of the *fdxN* and *nifD* excision elements showed that both mutants were defective for excision of the *fdxN* element in heterocysts after nitrogen deprivation, however, they both excised the 11-kb *nifD* element normally (Fig. 3). These results indicate that failure to excise the *fdxN* element blocks the downstream transcription of the *nifD-gfp* reporter and presumably all *nif* genes downstream of the element. The results suggest that sequences upstream of the *fdxN* element are required for expression of the downstream *nif* genes including *nifHDK*, and that an independent *nif* promoter is not present in the region immediately upstream of *nifH*.

Evidence that P_{nifB} is required for expression of the *nifHDK* genes. During our studies, the Thiel laboratory determined that in *A. variabilis*, a promoter upstream of *nifB1* is the primary promoter for the entire *nif1* gene cluster and that RNA processing was responsible for the very stable *nifH1* transcript and its previously reported 5' end (Ungerer et al. 2010). They later showed that RNA processing and secondary structure were necessary for *nifH1* transcript stability (Pratte and Thiel 2014; Pratte et al. 2015). From our results in *Anabaena* PCC 7120, we had hypothesized that transcription of the *nifHDK* genes might originate at the distant upstream *nifB* promoter and that failure to excise the *fdxN* element was blocking transcription from P_{nifB}. RNA-seq data for *Anabaena* PCC 7120 was consistent with this hypothesis because there was no evidence for internal TSSs within the *nif* gene cluster except for the *nifH* and *fdxH* upstream regions (Flaherty et al. 2011), which we found in this work do not produce promoter activity. In RNA samples isolated 21 hours after nitrogen deprivation (Flaherty et al. 2011), RNA-seq reads covering the *nif* gene cluster begin at P_{nifB} and continue past *nifK* to the end of the *fdxH* gene with only the *nifH* and *fdxH* upstream regions showing evidence of a potential TSS or mRNA processing site, which is seen in the RNA-seq reads a large number of transcript ends starting at a specific nucleotide followed by a consistent large number of downstream reads.

To determine if transcription of the *nifD-gfp* reporter could be driven by upstream proximal sequences, we modified pAMKK2 to contain a region of homology for the *gvp* locus to make plasmid pAMKK3. We then constructed a strain in which pAMKK3 had inserted into the ectopic *gvp* locus in the chromosome. Consistent with our other results, a representative clone, KKC1, did not show GFP fluorescence in heterocysts after

nitrogen deprivation, which indicates that this 2274-bp region lacks a strong independent promoter (Fig. 2C). However, this does not exclude the presence of a weak promoter in this region, such as was suggested by RNA-seq data from *Anabaena* PCC 7120 that showed evidence of a TSS in the *nifU* region (Flaherty et al. 2011). Also, in *A. variabilis*, weak promoters were identified within the *nifU1* ORF (Pratte and Thiel 2014).

A developmentally regulated promoter is present in the intergenic region upstream of *nifB*. A putative *nifB* TSS has been mapped at -283 relative to the *nifB* start codon (Mulligan and Haselkorn 1989). Analysis of RNA-seq data identified a potential TSS at position -282 bp relative to the *nifB* start codon (Flaherty et al. 2011). To determine if the region containing this putative TSS has an active promoter, we constructed a P_{nifB}-*gfp* transcriptional fusion in pAM1956 using the intergenic region between *nifB* and its upstream gene *asl1518*. The resulting strain, KKC2, showed heterocyst-specific GFP fluorescence when grown without a source of combined nitrogen (Fig. 4A), which indicates that a developmentally regulated *nif* promoter lies within the intergenic region between *nifB* and *asl1518* in *Anabaena* PCC 7120.

***nifH2* promoter**. *Anabaena* PCC 7120 contains a second copy of *nifH*, *nifH2* (*alr0874*), that is distant from the main cluster of *nif* genes (Haselkorn 1986). The *nifH2* 5' upstream region is 1513 bp and the upstream *asl0873* gene is divergently transcribed. Analysis of RNA-seq data showed that *nifH2* is strongly upregulated after nitrogen deprivation from a putative TSS at -1007 bases upstream of the ATG start site (Flaherty et al. 2011). To determine if the promoter for the *nifH2* gene is located in the upstream intergenic region, we constructed a *nifH2-gfp* transcriptional fusion in

pAM1956 containing this intergenic region and transferred the reporter plasmid into *Anabaena* PCC 7120. The resulting strain, KKC26, showed strong heterocyst-specific GFP fluorescence when grown diazotrophically (Fig. 4B). These results show that the mapped *nifH2* TSS in the upstream intergenic region is driven by a strong developmentally regulated *nif* promoter.

DISCUSSION

Although the *nif* genes in *Anabaena* PCC 7120 were identified over 30 years ago, our understanding of the molecular and biochemical mechanisms that govern the regulation of these genes is still limited. Here, we report the use of *gfp* reporters in *Anabaena* PCC 7120 to study transcription of the *nifHDK* genes and show that excision of the *fdxN* DNA element in heterocysts is required for expression of a *nifD-gfp* reporter inserted into the native site on the chromosome. This result shows that there is no functional promoter in the region immediately upstream of the *nifHDK* gene cluster and therefore indicates that transcription originates at the far-upstream *nifB* promoter.

Although previous studies had identified a putative *nifH* TSS in *Anabaena* PCC 7120 at 123 bp upstream of the *nifH* start codon (Haselkorn et al. 1983), we found that transcriptional fusions of the *nifU-nifH* intergenic region and sequences up to 500 bp upstream of *nifH* on shuttle plasmids did not produce expression of *nifH-gfp* reporter fusions. In addition, we constructed a *gfp* transcriptional fusion with the upstream region of the *fdxH* gene but failed to detect developmentally regulated expression (data not shown). However, we succeeded in obtaining developmentally regulated *nifD-gfp* reporter expression by constructing a *gfp* reporter fusion to the *nifD* ATG start codon at

366 the native locus in the chromosome (strain AMC1774), which showed strong heterocyst-
367 specific expression of GFP fluorescence.

368 A UV-induced mutant of strain AMC1774 was obtained that was unable to grow
369 diazotrophically and did not show GFP fluorescence in heterocysts after nitrogen
370 deprivation. This mutant, KKC3, was complemented by a plasmid carrying *xisF* and the
371 flanking gene *alr1460*, suggesting that the original mutant was defective in excision of
372 the *fdxN* DNA element during heterocyst differentiation. We also obtained a
373 phenotypically similar strain, KKC4, which is the AMC1774 reporter strain carrying
374 expression library clone 1824-1. PCR analysis of mutant KKC3 and strain KKC4 found
375 that both strains failed to excise the *fdxN* element from the chromosome. These results
376 indicate that excision of the 59,428-bp element is required for downstream *nifHDK*
377 transcription and strongly suggest that a promoter upstream of the *fdxN* element is
378 required for transcription of the *nifHDK* genes. We expect that a similar situation occurs
379 in other heterocyst-forming cyanobacteria. A survey found 101 interruption elements in
380 28 of 38 heterocyst-forming cyanobacteria with sequenced genomes, with the majority
381 of the DNA elements located within nitrogen fixation and hydrogenase genes (Hilton et
382 al. 2016).

383 The expression library plasmids that complemented the dark UV mutant of
384 AMC1774 and clone 1824-1, which produced a dark clone of AMC1774, both contain
385 the *xisF* and *alr1460* genes and appear to be identical plasmids from the original library.
386 In the first case, we suspect that complementation of the UV mutant resulted from
387 normal excision of the *fdxN* element. For the overexpression of the *xisF* and *alr1460*
388 genes from the 1824-1 plasmid in KKC4, we suspect that we obtained a clone with a

389 partial or aberrant DNA rearrangement of the *fdxN* element, or that the 1824-1 plasmid
390 was somehow interfering with normal rearrangement because of the over expression of
391 the *alr1460* gene.
392 To determine if a second promoter was present in the *Anabaena* PCC 7120 *nifU*
393 ORF, we tested for expression of the *nifD-gfp* reporter construct containing a 2274-bp
394 fragment upstream of *nifD* integrated into the ectopic *gvp* locus in the chromosome.
395 This strain did not show GFP fluorescence in heterocysts after nitrogen deprivation,
396 which indicates that there is not a strong *nif* promoter in this region.
397 We showed that reporter fusions containing the intergenic region upstream of *nifB*
398 transcriptionally fused to *gfp* on pAMKK4 showed GFP fluorescence specifically in
399 heterocysts, demonstrating that this region contains a developmentally regulated
400 promoter. RNA 5' end mapping and RNA-seq data for *Anabaena* PCC 7120 identified 5'
401 ends at -282 and -283 bp, respectively, upstream of the *nifB* ORF (Mulligan and
402 Haselkorn 1989; Flaherty et al. 2011). Taken together, these results indicate that
403 transcription of the *nifB-fdxN-nifSUHDK* genes, and potentially all of the downstream *nif*
404 gene, originates from the promoter upstream of *nifB*.
405 Previous data have shown significantly lower levels of transcripts from the *nifB-*
406 *fdxN-nifSU* genes compared to the *nifHDK* genes (Mulligan and Haselkorn 1989). A
407 1.75-kb band in northern blots thought to be the *nifB* transcript was detected, however,
408 the levels were much lower than those of the *nifH* transcripts (Mulligan and Haselkorn
409 1989). The expected larger *nifB-fdxN-nifSU* transcript was not detected (Mulligan and
410 Haselkorn 1989; Golden et al. 1991). We hypothesize that similar to *A. variabilis*, the
411 difference in transcript levels is due to higher stability of the *nifHDK* transcripts

412 compared to the *nifB-fdxN-nifS-nifU* transcripts. The presence of stem-loop structures
413 close to the 5' or 3' ends of the mRNA has been shown to increase stability of
414 transcripts (Newbury et al. 1987; Kennell 2002; Kushner 2002). We examined the 5'
415 upstream region of *nifH* in *Anabaena* PCC 7120 for secondary structures using mfold
416 (Zuker 2003). This region showed a potential secondary structure with a stem-loop that
417 begins at -123 bp relative to the *nifH* start codon and is likely to contribute to the stability
418 of the *nifHDK* transcripts (Fig. 5). As previously noted (Ungerer et al. 2010; Pratte et al.
419 2015), the DNA sequences for the *nif* gene cluster in *Anabaena* PCC 7120 and the *nif1*
420 cluster in *A. variabilis* are very similar, and the *Anabaena* PCC 7120 stem-loop is nearly
421 identical to the stem-loop sequence near the 5' end of the *nifH1* transcript in *A.*
422 *variabilis*. An mRNA 5' end for *nifH* was previously mapped at position -123 bp relative
423 to the start codon (Haselkorn et al. 1983; Jackman and Mulligan 1995). The 5' end at
424 this position corresponds to the predicted processed end at the edge of the stem-loop
425 rather than a *de novo* transcription start, and stable *nifHDK* transcripts are likely to be
426 cleavage products of a larger *nifB-fdxN-nifSUHDK* transcript, which may also contain
427 additional downstream genes.
428 We also show in this work that the second copy of the *nifH* gene, *nifH2*, is driven by
429 a developmentally regulated promoter that is located proximal to the gene in its
430 immediate upstream intergenic region. In addition, we used *gfp* reporter constructs to
431 show that developmentally regulated promoters for two heterocyst-specific genes, *hglD*
432 and *hglE*, are located in the intergenic region immediately upstream of each gene (data
433 not shown). *hgl* genes are involved in the production of the heterocyst glycolipid layer
434 and are upregulated during the middle stages of heterocyst differentiation. The use of

435 *gfp*-reporter fusions to demonstrate the activity of developmentally regulated promoters
436 presented in this study will help lead to a better understanding of heterocyst
437 development.
438
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444

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543
544

545 Table 1. Strains and plasmids used in this study

Strain	Characteristics	Source or reference
***E. coli* strains**		
AM1358	Strain DH10B carrying conjugal helper plasmid pRL623; Cm^r	(Elhai et al. 1997; Liu and Golden 2002)
AM1359	Strain DH10B carrying helper plasmid pRL623 and conjugal plasmid pRL443; Cm^r Ap^r Tc^r	(Elhai et al. 1997; Yoon and Golden 1998)
AM1460	Strain DH10B carrying conjugal plasmid pRK2013; Km^r	(Liu and Golden 2002)
AM1824 Library	Expression library of *Anabaena* PCC 7120 random DNA fragments expressed from the *rbcL* promoter in pAM1011; Km^r Nm^r	(Liu and Golden 2002)
***Anabaena* sp. strains**		
AMC1774	*Anabaena* PCC 7120 carrying pAMKK2 recombined into the chromosome at the native *nif* locus	This study
KKC1	*Anabaena* PCC 7120 carrying pAMKK3 recombined into the chromosome at the *gvp* neutral-site locus	This study
KKC2	*Anabaena* PCC 7120 carrying pAMKK4	This study
KKC3	GFP-dark mutant of AMC1774 obtained by UV mutagenesis	This study
KKC4	Strain AMC1774 carrying expression library plasmid 1824-1	This study
KKC5	*Anabaena* PCC 7120 carrying pAMKK5	This study

KKC6	*Anabaena* PCC 7120 carrying pAMKK6	This study
KKC25	*Anabaena* PCC 7120 carrying pAMKK25	This study
KKC26	*Anabaena* PCC 7120 carrying pAMKK26	This study
AMC444	*Anabaena* PCC 7120 carrying pAM882 containing 834-bp internal fragment of *xisF*	(Carrasco et al. 1994)
Plasmids		
1824-1	Expression library plasmid clone containing ORFs *alr1459–alr1460*	This study
pAM1956	Shuttle vector pAM505 containing promoterless *gfpmut2* with unique upstream cloning sites SalI, SacI, KpnI, and SmaI; Km^r Nm^r	(Yoon and Golden 1998)
pAMKK1	Suicide reporter vector containing *gfpmut2* from pAM1956 in suicide vector pRL277; Km^r Nm^r	This study
pAMKK2	Suicide vector pAMKK1 containing 2274-bp fragment upstream of *nifD* translationally fused to *gfp*; Km^r Nm^r	This study
pAMKK3	Neutral-site plasmid modified from pAMKK2 by insertion of 1.5-kb *gvp* gene fragment; Km^r Nm^r	This study
pAMKK4	Shuttle vector pAM1956 containing a 936-bp fragment upstream of *nifB* transcriptionally fused to *gfp*; Km^r Nm^r	This study

pAMKK5	Shuttle vector pAM1956 containing 221-bp fragment upstream of *nifH* transcriptionally fused to *gfp*; Km^r Nm^r	This study
pAMKK6	Shuttle vector pAM1956 containing 350-bp fragment upstream of *nifH* transcriptionally fused to *gfp*; Km^r Nm^r	This study
pAMKK25	Shuttle vector pAM1956 containing a 500-bp fragment upstream of *nifH* transcriptionally fused to *gfp*; Km^r Nm^r	This study
pAMKK26	Shuttle vector pAM1956 containing a 500-bp fragment upstream of *nifH2* transcriptionally fused to *gfp*; Km^r Nm^r	This study
pRL277	Conjugal suicide vector; Sp^r, Sm^r	(Elhai et al. 1997)

546

547

548 Table 2. Primers used in this study

Primer	Sequence	Source or reference
KKO-1	AGTAAAGGAGAAGAACTTT	This study
KKO-2	CCCAGCTTGCATGCCTGCAG	This study
KKO-3	ATTAGGGAATAGGAAGAAGC	This study
KKO-4	CATTATCTGCCTCAGTG	This study
KKO-5	ATTAGGGAATAGGAAGAAGC	This study
KKO-6	CATTATCTGCCTCAGTG	This study
KKO-7	CAATTACTAATTACCACATC	This study
KKO-8	TGAATTTCGCAAATGTCGGT	This study
KK0-9	TAGTTAACAGTTAACAGTAAACC	This study
KK0-10	TATCTGTCTAATGTTTTCGTCA	This study
KKO-11	GGCGATATTGTCAAAGTAG	This study
KKO-12	TCTGTCTAATGTTTTCGTCAGTC	This study
KKO-15	ATTCCTAATTCTGGG	This study
KKO-16	TGTTCTCTTTTCCTG	This study

549

550

FIGURE LEGENDS

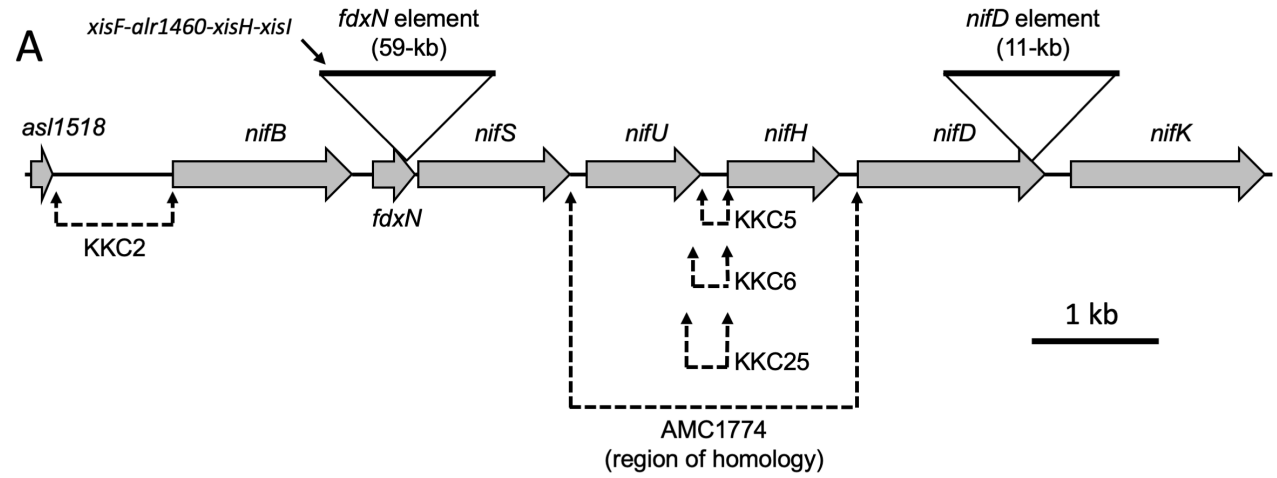
Fig. 1. (A) Schematic of a portion of the *nif* gene cluster in *Anabaena* sp. strain PCC 7120. Arrows and dotted lines indicate the regions used in the construction of GFP reporter plasmids and labels indicate the resulting reporter strains. The location of the *xisF-alr1460-xisH-xisI* genes on the left end of the *fdxN* element are shown. The *fdxN* and *nifD* DNA elements that excise from the chromosome in heterocysts are not drawn to scale. (B) Developmentally regulated GFP expression from heterocysts of strain AMC1774 in which a *nifD-gfp* reporter fusion is located at the native site in the chromosome. Left panel, bright-field image; middle panel, autofluorescence image of photosynthetic pigments; right panel, GFP fluorescence image. Arrowheads mark selected heterocysts. Images were captured 22 hours after nitrogen deprivation. Scale bar, 10 μm.

Fig. 2. Excision of the *fdxN* element is required for expression of the downstream *nif* genes, and an extended region upstream of the *nifHDK* gene cluster does not show promoter activity. Strains defective for excision of the *fdxN* element do not express the *nifD-gfp* reporter at the native locus: (A) UV-induced mutant strain KKC3 and (B) strain KKC4, which contains expression library plasmid 1824-1. (C) Strain KKC1 containing the *nifD* upstream region driving *gfp* integrated at the *gvp* neutral-site locus. Images are merged autofluorescence of photosynthetic pigments and GFP-reporter fluorescence images. Heterocysts were induced to differentiate by transfer of strains to BG-11_0 medium, which lacks a source of combined nitrogen. Images were collected 22 hours after nitrogen deprivation. Arrows mark selected heterocysts. Scale bars, 10 μm.

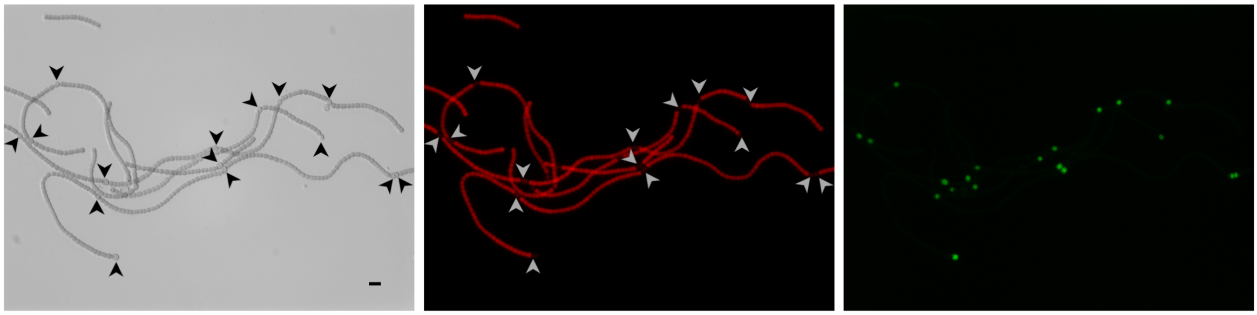
Fig. 3. PCR analysis of excision mutants KKC3 and KKC4. Lane 1 shows a DNA fragment that is amplified when the *fdxN* element is excised in the wild-type (WT) strain after nitrogen deprivation (BG-11_0). Lane 2 shows the absence of the amplified band in WT with a source of combined nitrogen (BG-11). Lanes 3 and 4 show a DNA fragment that is amplified by the excision of the *nifD* element under conditions of nitrogen deprivation in mutants KKC3 and KKC4, indicating that the *nifD* element was excised normally in these strains. Lanes 5 and 7 show the absence of the *fdxN* element excision band in the KKC3 and KKC4 under conditions of nitrogen availability (BG-11). Lanes 6 and 8 show the absence of the *fdxN* element excision band in KKC3 and KKC4 under conditions of nitrogen deprivation (BG-11_0), indicating that the *fdxN* element was not excised in these mutants. Marker lane contains a 1-kb ladder.

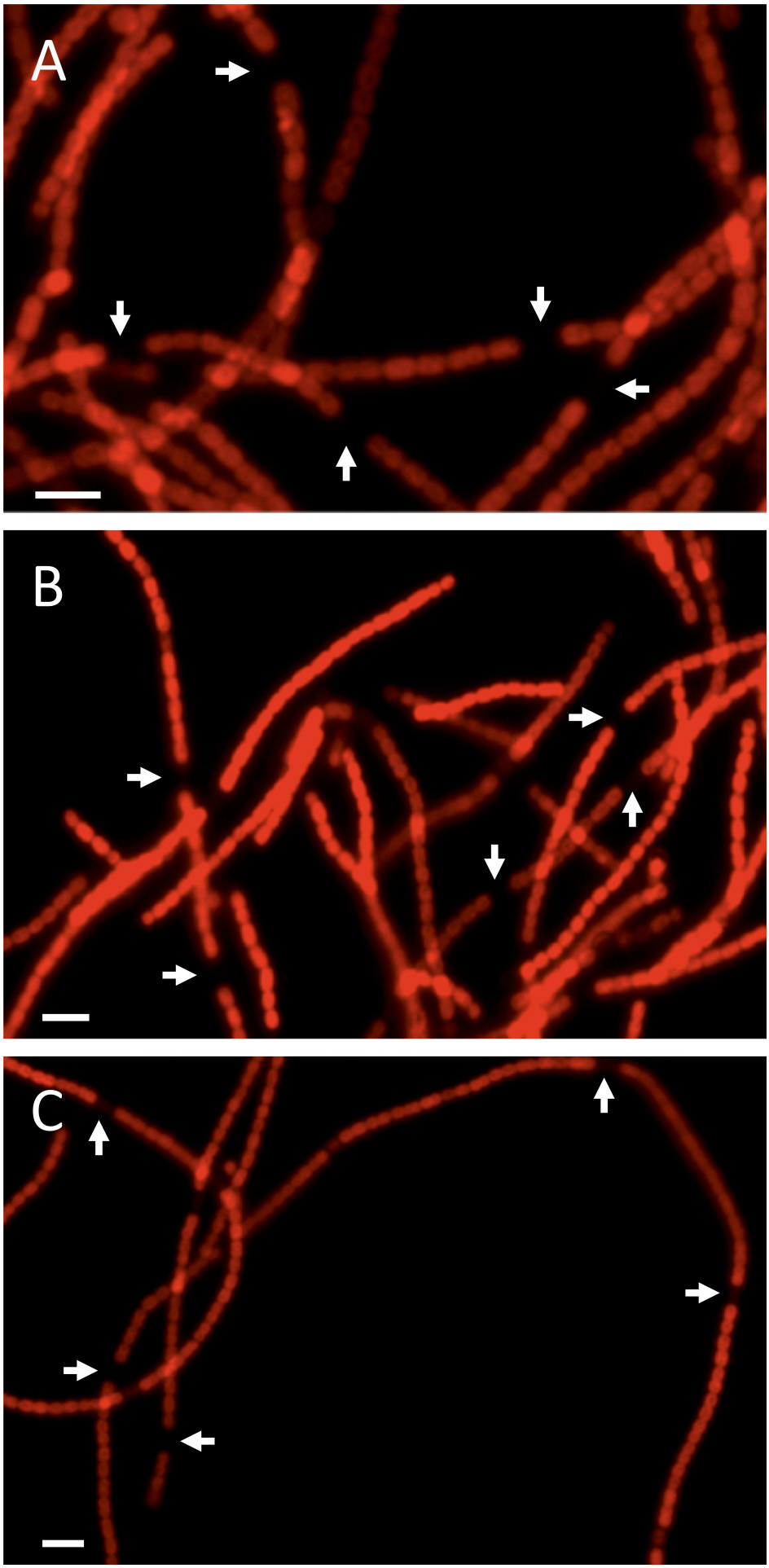
Fig. 4. GFP reporter fluorescence from strains containing *gfp* expressed from upstream regions of (A) *nifB*, strain KKC2 and (B) *nifH2*, strain KKC26. Left panels show bright-field images; right panels show the corresponding merged GFP and autofluorescence images. Heterocysts were induced to differentiate by transfer of strains to BG-11_0 medium. Images were collected at 22 hours after nitrogen deprivation. Scale bar, 10 μm.

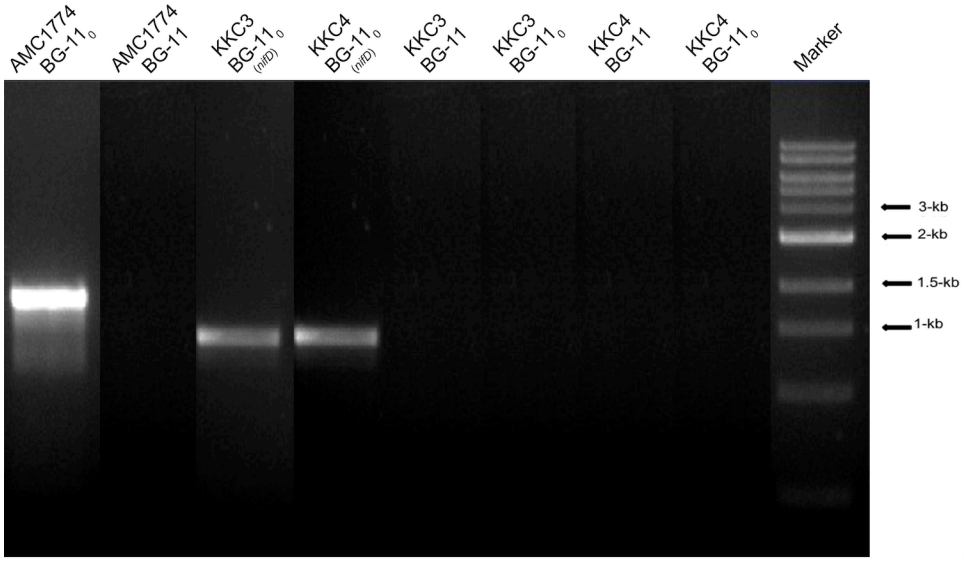
Fig. 5. Predicted stem-loop structure at the 5' end of the stable *nifH* transcript in *Anabaena* PCC 7120.

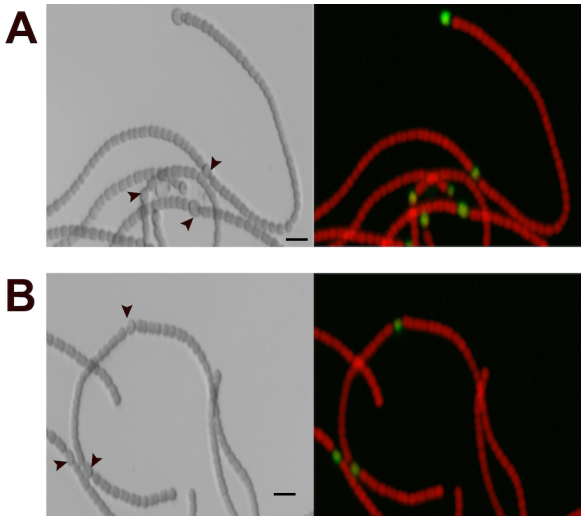

A
xisF-alr1460-xisH-xisI
fdxN element
(59-kb)
nifD element
(11-kb)
asl1518
nifB
nifS
nifU
nifH
nifD
nifK
fdxN
KKC2
KKC5
KKC6
KKC25
1 kb
AMC1774
(region of homology)

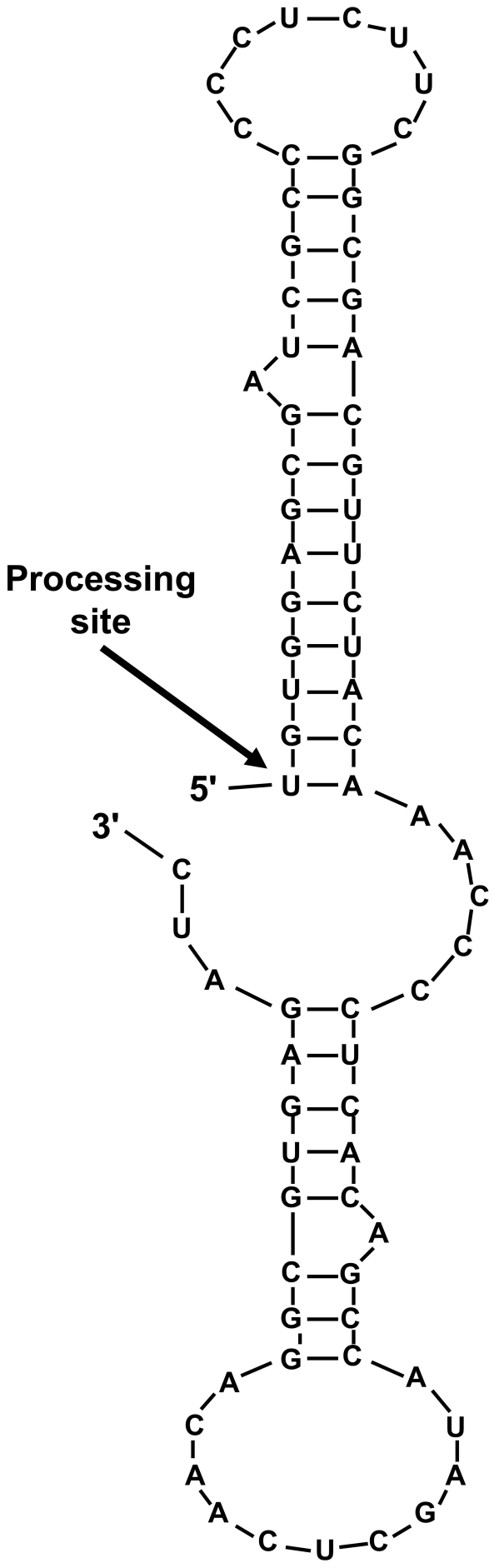
B




A
B
C


AMC1774 BG-11$_0$
AMC1774 BG-11
KKC3 BG-11$_0$ (*nifD*)
KKC4 BG-11$_0$ (*nifD*)
KKC3 BG-11
KKC3 BG-11$_0$
KKC4 BG-11
KKC4 BG-11$_0$
Marker
3-kb
2-kb
1.5-kb
1-kb


A
B


Processing
site
5'
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