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Vectors for Determining the Differential Expression of Genes in Heterocysts and Vegetative Cells of *Anabaena* sp. Strain PCC 7120

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Plasmid vectors were constructed to study promoters of the cyanobacterium *Anabaena* sp. strain PCC 7120. Plasmid pCCBSelect contains the promoterless reporter genes in the order *cat-nifHDK*. In pCCBSelect/a, the *nifHDK* operon precedes the *cat* gene. Putative promoter sequences were cloned into a polylinker region upstream of the reporter genes. Activity in heterocysts was determined by complementation of a strain containing a deletion of the *nifH* gene. Activity in vegetative cells was determined by measuring resistance to chloramphenicol. The promoter of the *nifHDK* operon was active only in heterocysts; the promoter of the *nifJ* gene was active only in iron-depleted medium; and the promoters of the *psbB* gene, the *ntcA* gene, and a newly found transcription factor gene were all active in both cell types.

Heterocysts are terminally differentiated cells specialized for nitrogen fixation and spaced at regular intervals along the filaments of some cyanobacteria. In *Anabaena* sp. strain PCC 7120, the interval of photosynthetic vegetative cells between heterocysts is approximately 10 cells. The heterocyst is structurally different from a vegetative cell and is surrounded by a double-layered envelope outside its cell wall.

Early experiments using cloned genes to study transcription during heterocyst development yielded results consistent with the simplest notion of transcriptional regulation: in heterocysts, genes for photosynthesis and carbon fixation should be turned off and genes for nitrogen fixation should be turned on. In nitrogen-replete cultures, transcripts of the *rbcLS* genes, encoding the enzyme RuBP carboxylase, were abundant while those of the *nifHDK* genes, encoding the subunits of nitrogenase, were absent. When combined nitrogen was removed and heterocyst differentiation was induced, the *rbcLS* transcripts vanished and the *nifHDK* transcripts became abundant (9).

This simple view of transcriptional regulation was complicated by studies of the *psbA* and *psbB* genes. These genes encode D1 and CP-47, components of photosystem II reaction centers. In differentiating cultures, photosystem II activity disappears, but the levels of *psbA* and *psbB* transcripts remain unchanged (4, 10, 12). Since heterocysts represent only 10% of the total cell population, this experiment did not directly address the level of mRNA for these genes in heterocysts.

Transcription at the single-cell level in *Anabaena* sp. has been measured by using *Vibrio* luciferase genes as reporters (5, 6, 18). The luciferase assay requires a long-chain aldehyde, $FMNH_2$ and O_2. The light produced by single cells can be measured by using a microscope fitted with a photon counter. This assay can distinguish genes expressed only in vegetative cells from those expressed in heterocysts, but quantitative measurements of gene expression in heterocysts with *lux* gene fusions are complicated by the limited permeability of heterocysts to O_2. Experiments with the *lux* reporter fused to various promoters showed that the *rbcL* promoter is indeed turned off in developing heterocysts and the *nifH* promoter is activated (6).

We present here an alternative system to measure promoter activity in heterocysts and in vegetative cells. One component of the system is the *nifHDK* operon. Since additional *nif* genes are required for cofactor synthesis and nitrogenase must be protected from O_2, the *nifHDK* operon yields active nitrogenase only in heterocysts. The promoter to be examined is fused to a promoterless *nifHDK* operon and a plasmid carrying this fusion is introduced into an *Anabaena* strain lacking the *nifH* gene. The ability to grow on N_2 as the sole nitrogen source serves as the basis for detection of promoter activity in heterocysts. In addition, DNA sequences that function as promoters can drive transcription of the *cat* gene. Resistance to chloramphenicol (CAM) then provides a measure of promoter function in the vegetative cells.

Vector constructions. The promoter test vectors are called pCCBSelect and pCCBSelect/a, shown in Fig. 1 and 2. The vectors contain origins for replication in *Escherichia coli* and in *Anabaena* sp. strain PCC 7120 and a transfer origin for mobilization during conjugation. A text description of the constructions runs to two pages. This description, as well as drawings of all the intermediates in the constructions, is available from the authors and can be found in reference 2.

Promoter testing. Each promoter construct in pCCBSelect and pCCBSelect/a was transferred by conjugation into *Anabaena* strain LW-1, a gift from J. Golden (7). Strain LW-1 contains a 1.68-kb deletion of the *nifH* gene and its promoter, such that it cannot revert and it cannot recombine with a promoterless *nifHDK* operon on a plasmid to reconstruct a functional operon. The deleted segment is replaced by a Spc^r-Str^r cassette (5). Neomycin- and spectinomycin-resistant colonies were grown in 100 ml of BG-11 containing 30 μg of neomycin per ml and 2 μg of spectinomycin per ml and washed with BG-11 without combined nitrogen. One set was plated on K&M plates containing neomycin (30 μg/ml), spectinomycin (2 μg/ml), CAM (10 μg/ml), and NO_3. The other was plated on K&M plates containing neomycin (30 μg/ml), and spectinomycin (2 μg/ml), omitting combined nitrogen and CAM. Two controls were added to each set. One consisted of the mutant strain LW-1 alone, while the other contained exconjugants of

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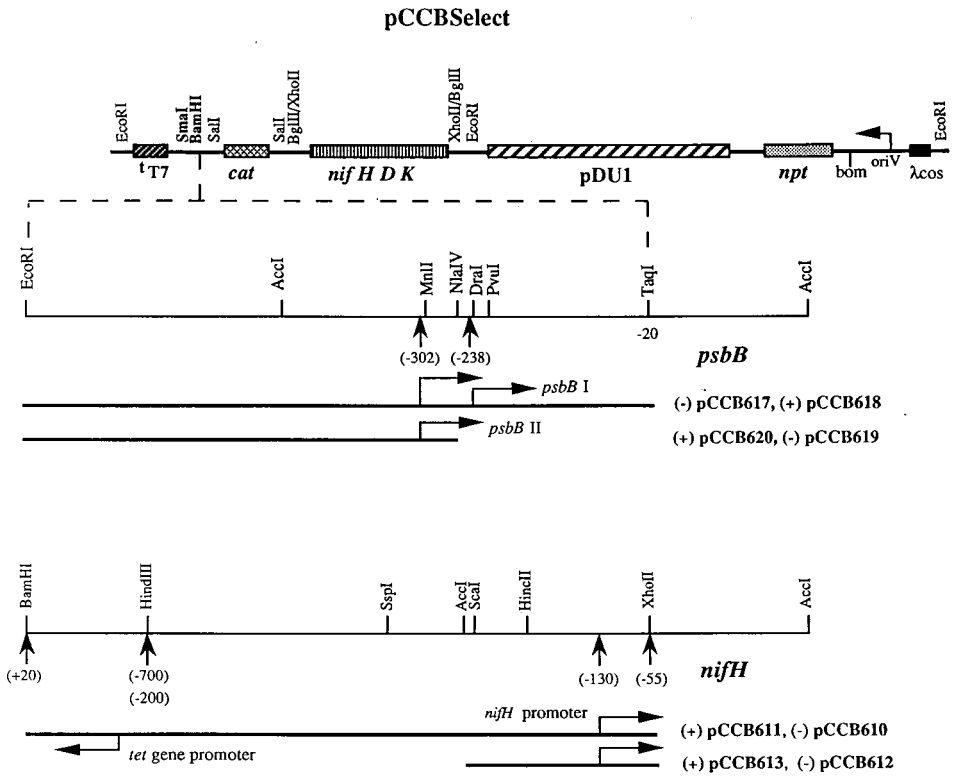

FIG. 1. Cloning of promoters into pCCBSelect. Construction of pCCBSelect and pCCBSelect/a is described in reference 2. The vectors can replicate in both *E. coli* and *Anabaena* sp. strain PCC 7120, are selectable with neomycin, have a multiple cloning site preceded by a T7 terminator for cloning of promoters, have a promoterless *cat* resistance gene to test promoter activity in vegetative cells, and have a promoterless *nifHDK* operon to allow selection for heterocyst gene expression when used in LW-1 (a strain deleted of its *nifH* gene and promoter) and a mobilization site for conjugative transfer into *Anabaena* sp. strain PCC 7120. Promoter fragments obtained by PCR or subcloning from larger clones were inserted into the vector as shown. In addition to the *nifH* promoter, pCCBSelect was used to clone the entire *psbB* promoter region and a deletion fragment of it containing only one promoter. The start sites for these promoters are shown by horizontal arrows. Numbers refer to the distance in nucleotides from the translational start of the gene. Both forward (+) and reverse (−) orientations of a promoter fragment were tested. The longer *nifHDK* fragment has the *tet* gene promoter initiating transcription when the fragment is in the reverse orientation.

the vectors pCCBSelect or pCCBSelect/a. Plates were incubated for 2 weeks, and growth was scored under the specified conditions. In each case, we verified, with restriction digests, that there had been no changes in the promoter-containing fragments resulting from cloning, conjugation, propagation in *Anabaena* sp. strain PCC 7120, or reisolation in *E. coli*. Changes were noted, however, in the survivors of conjugations involving the *rbcL*, *glnA*, and *hetR* promoters in pCCBSelect/a.

Background activities. The pCCBSelect vector alone did not complement the deletion mutation in LW-1, indicating that the vector requires a promoter element to function in that test. pCCBSelect/a alone also did not complement the LW-1 mutation, but it yielded a basal level of 10-μg/ml CAM resistance. Cells carrying the pCCBSelect vector were sensitive to 10 μg of CAM per ml. LW-1 itself showed no revertant colonies able to fix nitrogen and no resistance to CAM at 10 μg/ml (see Table 2). The low-level resistance to CAM conferred by pCCB Select/a could be due to a weak promoter sequence near the end of the *nifHDK* operon that reads through into the *cat* gene in that construction.

The *psbB* promoters. Using the pCCBSelect system, we tested DNA fragments containing the promoters of the *psbB* gene for *nifH* complementation and CAM acetyltransferase activity (Table 1). A DNA fragment containing both promoters conferred resistance to 40 μg of CAM per ml in the forward orientation. Promoter II constructs provided resistance to 10 μg of CAM per ml only in the forward orientation. A construct containing both promoters I and II was able to complement LW-1, the *nifH* deletion mutant. Heterocyst morphology and average filament length appeared to be normal in these strains. Constructs containing only promoter II did not complement LW-1, similar to the promoterless pCCBSelect control exconjugants. These results are consistent with previous measurements of CAM acetyltransferase protein driven by these two promoter fragments. When promoter II alone is present, the CAM acetyltransferase level is only 15% of that seen when both I and II are present (11). The lower level of expression driven by promoter II alone is not sufficient to complement the *nifH* deletion in strain LW-1.

The *nifH* promoter. The *nifH* promoter was capable of complementing the deletion mutant, LW-1, by driving the expression of the *nifHDK* operon located on the plasmid pCCB Select. Both the 700- and 300-bp *nifH* promoter fragments complement mutant LW-1. The 300-bp *nifH* promoter fragment did not confer resistance to CAM during vegetative growth, appropriate for a promoter shown to be active only in heterocysts (6); however, the 700-bp promoter fragment conferred resistance to CAM in either orientation up to at least 20 μg/ml (Table 1). Previous experiments using *lacZ* as a reporter had shown that this 700-bp fragment had substantial constitutive promoter activity in *E. coli* (14). These results indicate that an upstream sequence could act as a promoter in vegetative cells. This sequence is inside the coding region of the *nifU* gene. When the 700-bp *nifH* promoter fragment is in the re-

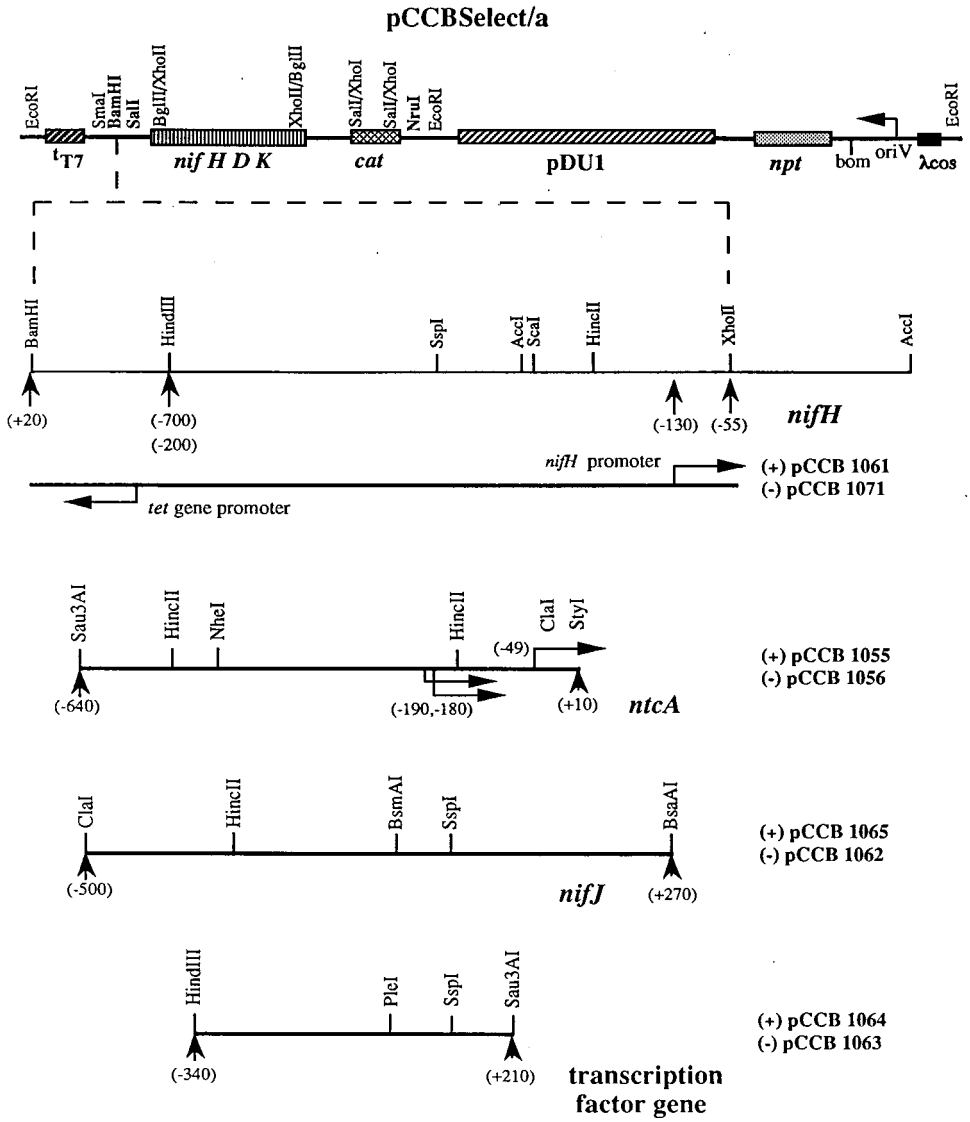

FIG. 2. Cloning of promoters into pCCBSelect/a. pCCBSelect/a was used to clone the *rbcL*, *glnA*, *ntcA*, *nifJ*, and *nifH* promoters and a transcription factor gene promoter. Orientation with regard to the start site of translation is shown next to the plasmid names. Sizes of the fragments are shown relative to the start of translation as well. The transcription starts for the *ntcA* gene were communicated privately (13).

TABLE 1. Growth of *Anabaena* strain LW-1 carrying pCCBSelect derivatives[a]

Plasmid	Promoter orientation	Known promoter(s) operating	Growth on N^- medium	Growth on N^+ medium with CAM at μg/ml:		
				10	20	40
pCCB617	−	*psbB*; None	No	No	No	No
pCCB618	+	*psbB*; I, II	Yes	Yes	Yes	Yes
pCCB619	−	*psbB*; None	No	No	No	No
pCCB620	+	*psbB*; II	No	Yes	No	No
pCCB610	−	*tet* gene	No	Yes	Yes	Yes
pCCB611	+	700-bp *nifH* upstream	Yes	Yes	Yes	No
pCCB612	−	300-bp *nifH* upstream	No	No	No	No
pCCB613	+	300-bp *nifH* upstream	Yes	No	No	No

[a] Culture conditions are those described previously (2). Promoter orientation: +, forward direction; −, reverse.

verse orientation, the *tet* gene promoter (from pBR322) is active (Fig. 1). This construct confers resistance to CAM up to 40 μg/ml (Table 1). pCCBSelect/a constructs containing the 700-bp *nifH* promoter with the 200-bp *tet* gene promoter produced results identical to those seen with pCCBSelect (Table 2).

The *ntcA* and *nifJ* promoters. The promoter of the *ntcA* gene was tested with the pCCBSelect/a system (Table 2). This gene encodes a transcription factor required for expression of both vegetative cell and heterocyst genes. It binds to the promoter regions of the *rbcL*, *glnA*, *nifHDK*, and *xisA* genes (16, 17). The *ntcA* promoter weakly complemented the LW-1 mutant, that is, this strain grew more slowly on N^- K&M plates than one containing the *nifH* promoter construct and required 1 week longer to green. The *ntcA* construct conferred resistance to 20 μg of CAM per ml. In the opposite orientation, this promoter fragment conferred resistance to 10 μg of CAM per ml, equal to the basal level conferred by the vector alone, and did not complement the LW-1 mutation. These results are consistent

TABLE 2. Growth of *Anabaena* strain LW-1 carrying pCCBSelect/a derivatives[a]

Plasmid	Promoter orientation	Known promoter(s) operating	Growth on N^- medium	Growth on N^+ medium with CAM at μg/ml:		
				10	20	40
pCCB1071	−	*nifH*; *tet* gene +	No	Yes	Yes	Yes
pCCB1061	+	700-bp *nifH* upstream	Yes+++	Yes	Yes	No
pCCB1056	−	*ntcA* upstream	No	Yes	No	No
pCCB1055	+	*ntcA* upstream	Yes+	Yes	Yes	No
pCCB1062	−	*nifJ* upstream	No	Yes	No	No
pCCB1065	+	*nifJ* upstream	(−Fe) Yes++	Yes	Yes	No
pCCB1063	−	Transcription factor	No	Yes	No	No
pCCB1064	+	Transcription factor	Yes+	Yes	Yes	Yes
Strain LW-1		None	No	No	No	No
pCCBSelect/a		None	No	Yes	No	No

[a] Promoter orientation symbols are as in Table 1. Plus signs in the growth column indicate vigor, with +++ approaching wild-type growth. The background resistance to CAM provided by the vector alone is discussed in the text.

with the known promoters determined by primer extension and shown in Fig. 2 as horizontal arrows on the map of the *ntcA* promoter fragment (13).

The *nifJ* promoter region in pCCBSelect/a was unable to complement the LW-1 mutation under normal N^- conditions. However, under conditions of iron limitation the *nifJ* construct did complement LW-1. Without iron in the medium, it took only 2 days longer than the *nifH* pCCBSelect/a construct to complement LW-1. Previous Northern (RNA) analysis indicated that *nifJ* is transcribed only when iron is limiting in the medium, but is essential for nitrogen fixation, indicating that it is also expressed in the heterocyst (1). Thus, the results seen with the cell-type-specific reporter vector are consistent with the known properties of *nifJ* expression.

The promoter of a putative transcription factor gene. In work reported elsewhere, we describe a method for cloning cDNA fragments corresponding to mRNAs expressed at different times during heterocyst differentiation (3). One of the cDNAs corresponded to the gene encoding sucrose synthase. The cDNA was used to clone a chromosomal fragment containing the complete *sucA* gene. Sequencing the ends of this fragment revealed an open reading frame encoding a polypeptide of 219 amino acids with considerable similarity to prokaryotic transcription factors (2). The best match to sequences in GenBank was to the NarL protein of *E. coli*, which is a regulator of transcription of genes in the nitrate utilization pathway (8, 15). The upstream region from this putative transcription factor gene from *Anabaena* sp. strain PCC 7120 was tested in the pCCBSelect/a vector system (Fig. 2 and Table 2). This construct complemented the LW-1 mutant strain as well as the *ntcA* promoter construct, and it conferred resistance to high concentrations of CAM. Thus, it appears to be active in both heterocysts and vegetative cells.

The *rbcL* and *glnA* genes. These two genes have strong promoters active in vegetative cells (9). It was not possible to recover exconjugants containing these promoters in pCCB Select/a, presumably because high-level ectopic expression of the *nifHDK* operon in vegetative cells is lethal. It was possible to recover some viable cells from the plates on which exconjugants were selected, but the plasmids extracted from these cells had all undergone rearrangements that deleted either the *nif* genes or parts of the promoters.

The vector pCCBSelect/a was created in order to make it easier to clone promoter fragments into sites upstream of the *nifHDK* operon. It contains additional restriction sites for cloning, relative to pCCBSelect, and also has sites to construct exonuclease III deletions of the promoters. Cloning of promoter fragments is facilitated in these constructs, because promoter-containing clones were detectable as larger *E. coli* colonies in a background of smaller more compact colonies. Constructs with only the *cat* gene as a reporter did not show colony size differences. We determined that the *lacZ* promoter controlled the expression of the *nifHDK-cat* operon in the intermediate vector pCCB627, and insertions of cyanobacterial promoter fragments generally decreased read-through from the *lacZ* promoter. We believe that abundant expression of *nifHDK* genes in *E. coli* is detrimental, resulting in a smaller colony.

The vectors can also be used for the detailed analysis of promoters. Each of the pCCBSelect/a constructs can be opened uniquely at a *Sal*I site upstream of the promoter fragment or at an *Xba*I site between the promoter fragment and the start of *nifHDK*, providing an entry for the construction of deletions entering the promoter from either the 5′ or the 3′ side. All of the pCCBSelect/a constructs are transcriptional fusions; the 55 nucleotides upstream of the *nifH* translational start, included in all of the constructs, contain termination triplets in all three reading frames (9). The copy number of the parent vector, pJL3, was determined to be rather close to the number of chromosomes (11). For the pCCBSelect/a constructs, copy number can be determined by probing a blot of a restriction digest with an oligonucleotide that recognizes the sequence just outside one endpoint of the *nifH* deletion in strain LW-1. Thus, it should be straightforward to extend the qualitative results reported here by using the vectors described.

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