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A Vector for Analysis of Promoters in the Cyanobacterium *Anabaena* sp. Strain PCC 7120

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A plasmid vector containing a multiple-cloning site followed by a promoterless chloramphenicol acetyltransferase (*cat*) gene, protected by transcription terminators and mobilizable by conjugation from *Escherichia coli* into *Anabaena* sp. strain PCC 7120, was constructed. The utility of the vector was shown by deletion analysis of the promoter region of the *Anabaena psbB* gene.

Anabaena sp. strain PCC 7120 is representative of a large number of cyanobacteria that grow in long filaments of cells that are identical as long as combined nitrogen is present in the growth medium. Deprived of combined nitrogen, specialized cells called heterocysts differentiate at regular intervals along each filament. Heterocysts fix nitrogen and provide it to neighboring vegetative cells in the form of the amide group of glutamine.

We wish to understand how *Anabaena* sp. regulates gene expression, which occurs principally at the level of transcription, during heterocyst differentiation. Characterization of the start sites for mRNA transcribed from the *nifHDK* and the *glnA* genes, encoding nitrogenase and glutamine synthetase, respectively, suggested that these genes may be transcribed by a modified form of RNA polymerase, which possibly requires additional transcription factors, in heterocysts (5, 10). On the other hand, genes transcribed in vegetative cells seem to have promoters that are similar in sequence and spacing to typical *Escherichia coli* promoters. In some of the latter cases, in vitro transcription with purified *Anabaena* RNA polymerase confirmed the start sites and, therefore, the promoter assignments made on the basis of S1 nuclease protection and primer extension analyses of in vivo RNA (9).

One gene of particular interest in this connection is *psbB*, encoding the 47-kDa chlorophyll-binding protein (CP-47) of photosystem II. This gene was shown to have two apparent promoters in vivo (8). Subsequent in vitro transcription of deleted templates confirmed both promoter assignments and showed further that each could initiate transcription in the absence of the other (7). Extension of this analysis required determination of the independent activity of each *psbB* promoter in vivo. For this purpose we constructed a promoter probe vector (pJL3) containing the following features: separate origins of replication that function in *E. coli* and in *Anabaena* sp. (3), a transfer origin for vector mobilization for conjugal transfer from *E. coli* to *Anabaena* sp. (3), a multiple-cloning site followed by a promoterless gene encoding chloramphenicol acetyltransferase (CAT) and a transcription terminator (4), and another transcription terminator preceding the multiple-cloning site (2). When promoter-containing DNA fragments are inserted into the multiple-cloning site in the correct orientation, transcription originating at the promoter will continue into the *cat* gene but will go no further.

A physical map of pJL3 is shown in Fig. 1. Construction of pJL3 began with pRL25C, which contains two replicons allowing replication in *E. coli* and in *Anabaena* sp., as well as the Neo^r gene and a *bom* region that allows conjugal transfer (3, 11). The pDU1 replicon is a plasmid isolated from a filamentous cyanobacterium related to *Anabaena* sp. The remainder of the unspecified sequences in pRL25c are derived from pBR322. A 600-bp *Eco*RI-*Pvu*II fragment from pTE103, containing the pUC8 multiple-cloning site and the early T7 terminator (4), was end filled by using the Klenow fragment of DNA polymerase, followed by ligation of *Eco*RI linkers. Digestion with *Eco*RI yielded a fragment that was cloned into the unique *Eco*RI site in pRL25C, creating plasmid pJL. Next, an 800-bp promoterless *cat* gene (Pharmacia LKB Biotechnology) was end filled and ligated to *Sal*I linkers. Digestion with *Sal*I yielded a fragment that was ligated into *Sal*I-digested pJL to give plasmid pJL1. Next, a 170-bp *Eco*RI fragment containing the *E. coli* rRNA gene terminator T1 (2) was isolated from plasmid pTLXT-11 (kindly provided by H. Deneer and G. Spiegelman). This fragment was end filled and cloned into *Sma*I-digested pJL1 to give plasmid pJL2. Finally, to destroy the *Bam*HI site outside the multiple-cloning site, pJL2 was partially digested with *Bam*HI, end filled, and religated; this yielded pJL3.

Maps of the *psbB* promoter-containing fragments are shown in Fig. 1. The complete nucleotide sequences of these fragments are given in Fig. 2. These fragments were cloned into the *Hin*cII or *Sma*I sites of pTE103, a vector used for in vitro transcription. Each of the promoter-containing derivatives of pTE103 was cut with *Eco*RI and *Hin*dIII, and the resulting fragments were end filled and ligated to *Bam*HI linkers. Digestion with *Bam*HI yielded fragments that could be cloned into the unique *Bam*HI site of pJL3.The correct orientation of the promoter-containing fragments could be deduced by plating the *E. coli* in which they are contained on LB agar containing chloramphenicol at 30 μg/ml and was subsequently verified by restriction analysis.

Each of the promoter-containing derivatives of pJL3 was transferred to *Anabaena* sp. strain PCC 7120 by conjugation (3). After several weeks on selective plates, single exconjugant colonies were suspended in 100 ml of BG-11 containing 30 μg of neomycin per ml and were allowed to grow for 1 week. The level of CAT protein was determined by using a kit from 5 Prime 3 Prime, Inc., Westchester, Pa. Cells from the 100-ml culture were collected, washed, resuspended in

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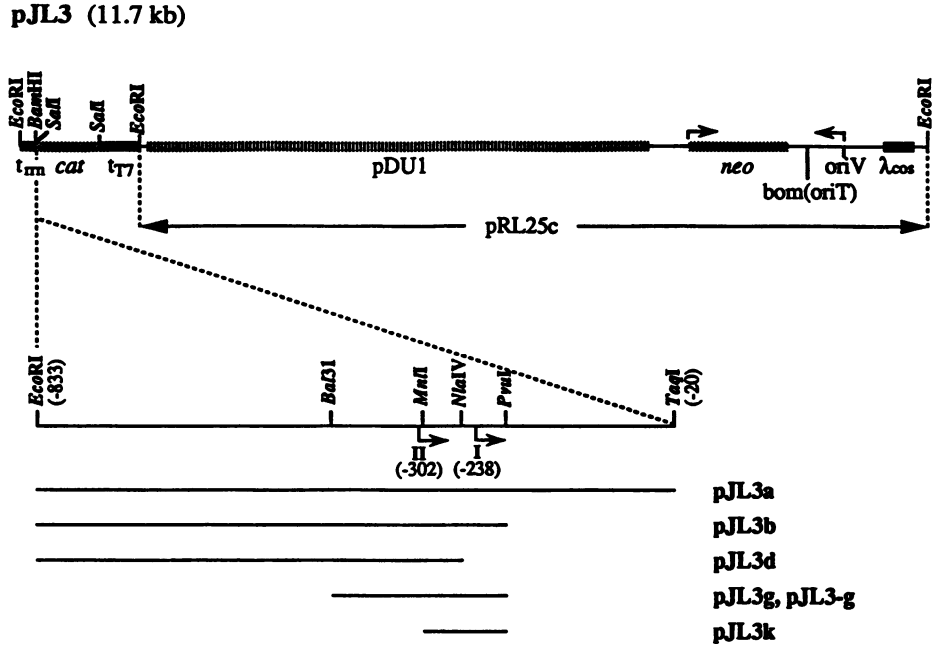

FIG. 1. Physical map of the promoter assay vector pJL3 and several of its derivatives. Construction of pJL3 and the origin of its elements are described in the text. The five promoter-containing fragments from the 5'-flanking region of the *Anabaena* sp. strain PCC 7120 *psbB* gene are shown below. Each of the fragments was cloned, in the orientation shown, into the *Bam*HI site of pJL3. In pJL3-g, the promoter-containing fragment is reversed. Numbers in parentheses refer to the distance, in base pairs, from the translation start of the *psbB* open reading frame. I and II refer to the start sites for transcription identified in vivo (8).

0.5 ml of dilution buffer, sonicated, and centrifuged for 15 min. Dilutions of the cleared sonicates were added to wells of microtiter plates coated with rabbit anti-CAT antisera. After being incubated and washed, the plates were further incubated with biotinylated anti-CAT antisera, which was then quantitated by incubation with streptavidin-conjugated alkaline phosphatase and substrate. Alkaline lysis minipreparations of plasmid DNA were made from each exconjugant and used to transform *E. coli* MC1061. From each of these transformants, plasmid DNA was prepared and the cloned inserts were sequenced. In each case, we verified that there had been no changes in the promoter-containing fragments resulting from cloning, conjugation, propagation in *Anabaena* sp., or reisolation in *E. coli*.

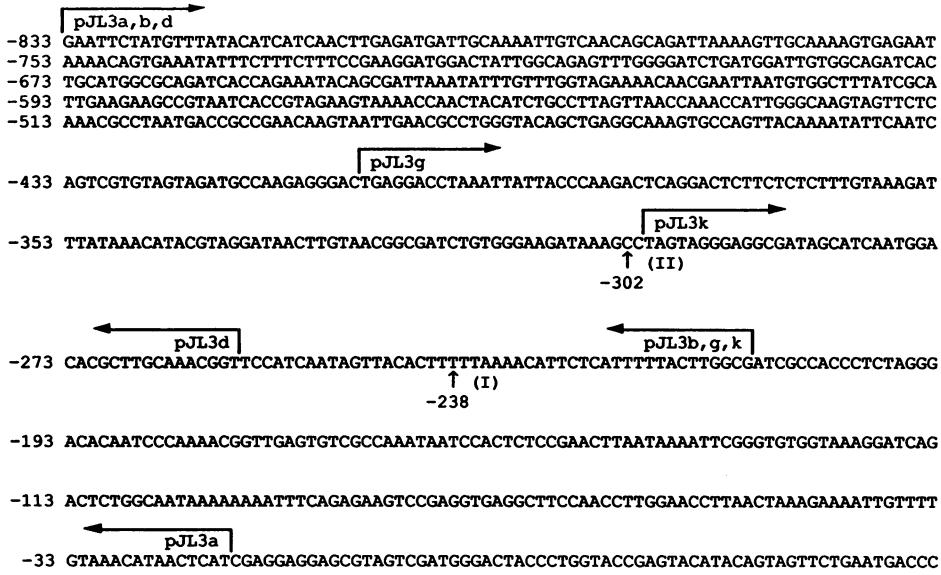

FIG. 2. Nucleotide sequence of the region containing the transcriptional start sites for the *psbB* gene. Numbering begins with the first A of the methionine codon. Vertical arrows indicate the two start sites determined for in vivo transcripts (8). Horizontal arrows indicate the endpoints of the deletions shown in Fig. 1 and show nucleotides included in each labeled clone.

TABLE 1. CAT protein levels in *Anabaena* strains carrying pJL3 derivatives

Plasmid	Promoter present (orientation)	Plasmid DNA level (%)[a]	CAT U[b] (pg/μg of protein)	CAT U/ plasmid copy
None			<10	
pJL3	None	100	<10	
pJL3a	I, II (+)	43	2,300	5,350
pJL3b	I, II (+)	107	690	645
pJL3d	II (+)	60	410	680
pJL3g	I, II (+)	88	300	340
pJL3k	I (+)	83	260	310
pJL3-g	I, II (−)	171	30	20

[a] The plasmid copy level was determined by dot blot as described in the text. The numbers give the observed level for each strain relative to the strain containing pJL3, which is listed as 100%. The absolute copy number for the latter strain is about one plasmid per chromosome, which is equivalent to 10 to 20 plasmids per cell. The copy number was estimated as follows: a dot blot containing 46 ng of pJL3a DNA bound about four times more counts per minute of the *cat* probe than did a dot blot containing 6.24 μg of total DNA from a pJL3a-containing strain of *Anabaena* sp. The size of the *Anabaena* sp. strain PCC 7120 chromosome is 6.4 Mb, and it has three megaplasmids totaling 0.7 Mb (1). The ratio of 12.5-kb pJL3a chromosomes to cellular and megaplasmid chromosomes is therefore as follows: 1/4 × (7,100/12.5) × (0.046/6.24) ≈ 1. *Anabaena* sp. strain PCC 7120 contains 10 to 20 chromosomes per cell (6).

[b] CAT levels were determined by ELISA as described in the text. Numbers shown are the means of four assays. No single measurement differed from the mean by more than 10%. See Fig. 1 for physical maps of the plasmids.

The results of the CAT measurements are collected in Table 1. The CAT level was normalized in two respects: with regard to total protein, of which the levels are similar in all the cultures, and with regard to plasmid copy number, the relative levels of which were determined by probing dot blots of total DNA with labeled *cat* DNA. The blots contained 1.5, 3, or 6 μg of total DNA from each of the plasmid-carrying strains. The probe was a random-primed 800-bp *cat* fragment. After locating the blots by autoradiography, each was cut out and counted. Plasmid copy number varied by a factor of 4 at most. The dot blots also contained CsCl-purified pJL3a DNA. Comparison of hybridization of the probe to pJL3a (12.5 kb) with hybridization to total DNA (7.1 Mb) indicated that the absolute plasmid copy number was approximately one per chromosome for pJL3a (Table 1). The controls (no plasmid, pJL3 alone, and pJL3 with the g fragment in opposite orientation [pJL3-g]) show that CAT expression requires a promoter-containing fragment, i.e., the vector functions as it should. The most striking result is the eightfold reduction in CAT level because of deletion of the 192-bp fragment between start site I and the translation start (compare pJL3a with pJL3b). This deletion has virtually no effect on in vitro transcription (7), so we believe that the deleted downstream nucleotides either stabilize the message or are required for efficient initiation of translation. Further deletion of start site I had no effect on CAT level (compare pJL3b with pJL3d). This too is unexpected, since at least half of the in vivo transcripts start at site I (8). Finally, a further twofold reduction in CAT is seen when 5′-flanking sequences which approach or include start site II are deleted. This result is also unexpected, because the big 5′ deletion of pJL3g had no consequence for in vitro transcription (7). Consistent with previous in vitro results, however, is the general observation that both promoter I and promoter II can function in the absence of the other.

We expect pJL3 to be useful both for the isolation of new vegetative cell promoter-containing fragments and for the isolation of promoters active in heterocysts. The absence of CAT expression from pJL3 indicates that there is no readthrough from plasmid promoters. Extracts can be frozen and stored at −20°C without changes in the levels of CAT detected by the enzyme-linked immunosorbent assay (ELISA) system. The system response is linear down to 50 pg of CAT per 200-μl sample, so very weak promoters should be detectable. Finally, slight further modification (elimination of the *Eco*RI site in the *cat* gene) will yield an *Eco*RI cassette that can easily be moved to any other vector.

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REFERENCES

1. **Bancroft, I., C. P. Wolk, and E. V. Oren.** 1989. Physical and genetic maps of the genome of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **171:** 5940–5948.
2. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *E. coli*. J. Mol. Biol. **148:**107–127.
3. **Elhai, J., and C. P. Wolk.** 1988. Conjugal transfer of DNA to cyanobacteria. Methods Enzymol. **167:**747–754.
4. **Elliot, T. E., and E. P. Geiduschek.** 1984. Defining a bacteriophage late promoter: absence of a −35 region. Cell **36:**211–219.
5. **Haselkorn, R., D. Rice, S. E. Curtis, and S. J. Robinson.** 1983. Organization and transcription of genes important in *Anabaena* heterocyst differentiation. Ann. Inst. Pasteur Microbiol. **134B:** 181–193.
6. **Herdman, M., M. Janvier, R. Rippka, and R. Y. Stanier.** 1979. Genome size of cyanobacteria. J. Gen. Microbiol. **111:**73–85.
7. **Lang, J. D.** 1990. Ph.D. dissertation. University of Chicago, Chicago, Ill.
8. **Lang, J. D., and R. Haselkorn.** 1989. Isolation, sequence and transcription of the gene encoding the photosystem II chlorophyll-binding protein, CP-47, in the cyanobacterium *Anabaena* 7120. Plant Mol. Biol. **13:**441–456.
9. **Schneider, G. J., N. E. Tumer, C. Richaud, G. Borbely, and R. Haselkorn.** 1987. Purification and characterization of RNA polymerase from the cyanobacterium *Anabaena* 7120. J. Biol. Chem. **262:**14633–14639.
10. **Tumer, N., S. J. Robinson, and R. Haselkorn.** 1983. Different promoters for the *Anabaena* glutamine synthetase gene during growth using molecular or fixed nitrogen. Nature (London) **306:**337–342.
11. **Wolk, C. P., Y. Cai, L. Cardemil, E. Flores, B. Hohn, M. Murry, G. Schmetterer, B. Schrautemeier, and R. Wilson.** 1988. Isolation and complementation of mutants of *Anabaena* sp. strain PCC 7120 unable to grow aerobically on dinitrogen. J. Bacteriol. **170:**1239–1244.