Chloroplast promoter driven expression of the chloramphenicol acetyl transferase gene in a cyanobacterium

Valdis A.Dzelzkalns, Geoffrey C.Owens and Lawrence Bogorad

Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA

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ABSTRACT

The putative promoter region of the chloroplast encoded ps2B gene (the gene encoding the 32kD herbicide binding B protein of photosystem II (1-4)) has been fused to a chloramphenicol acetyl transferase (CAT) gene that lacks its bacterial promoter and found to accurately initiate transcription from this promoter when introduced into the cyanobacterium, Anacystis nidulans R2 (or into E. coli). The chloroplast promoter-CAT fusion was introduced into the cells on a plasmid that contains plasmid replication origins for E. coli and Anacystis as well as a second antibiotic resistance marker. Cells transformed with corresponding vectors lacking the promoter region do not express CAT.

INTRODUCTION

The cyanobacteria are unique among photosynthetic prokaryotes in that, like higher plants, they perform oxygenic photosynthesis. However, unlike higher plants, these organisms present a number of advantages for studying the molecular biology of photosynthesis: photosynthetic mutants may be readily generated (5-6) and certain strains are genetically transformable (7-9). In order to examine whether chloroplast promoter sequences (and hence possibly chloroplast genes) can function in cyanobacteria, we have constructed a series of plasmids containing the chloramphenicol acetyl transferase (CAT) gene that lacks its bacterial promoter. Upstream of this promoterless gene we have inserted the putative promoter region of the chloroplast encoded ps2B gene (also designated psbA (10)) from an atrazine resistant biotype of Amaranthus hybridus (11,12). The plasmids also contain a second antibiotic resistance marker as well as origins of replication for plasmids in E. coli and the cyanobacterium Anacystis.

The ps2B promoter was chosen for this study because the ps2B gene is expressed at high levels in higher plant chloroplasts (1,2) and it is therefore believed that the gene is under the control of a strong promoter. ps2B has been isolated from various plant (10, 12-16), cyanobacterial (17), and

algal (18,19) sources and has been shown to code for the highly conserved B protein of photosystem II. In addition, a comparison of the nucleotide sequences upstream of higher plant ps2B genes indicates that the characteristic prokaryotic -10 and -35 promoter regions are also conserved. It has been recently shown in an in vitro system that these sequences are important for the transcription of this gene (15).

MATERIALS AND METHODS

Strains, Vectors, and Growth Conditions

The following plasmids were used in these experiments. Plasmid pMH1 (gift of L. Herrera-Estrella) contains the coding region of the CAT gene but has the endogenous promoter deleted (20). Plasmid pAH484 contains the ps2B gene isolated from an atrazine resistant biotype of Amaranthus hybridus (12). Plasmid pUC105 (gift of G.A. van Arkel and C.A.M.J.J. van den Hondel) is a shuttle vector that can replicate in E. coli and Anacystis (21). Plasmids pUC105-31, pMH1/HS484/4, pMH1/105-31/34, pMH1/105ori/19, pMH1/105ori/42, and pVAD1 and their construction are described in this report.

Axenic cultures of Anacystis nidulans (Synechococcus R2 6301, UTEX 625) were grown at 28°C in 80 ml of modified BG-11 media (BG-11 (22) supplemented with 2 mM HEPES pH 7.0). The cells were illuminated from a bank of six fluorescent cool white bulbs at approximately 3500 lux and were kept in suspension by agitation on rotary shakers.

Transformation of Anacystis Cells

Transformations were carried out as follows. Anacystis cells were grown (as described above) to an OD₇₃₀ of approximately 0.5 (about 2×10^8 cells/ml), collected by centrifugation at room temperature, resuspended in one quarter volume of growth medium, and recentrifuged. The pellet was resuspended in modified BG-11 medium at one-tenth of the original volume and 1 ml aliquots were pipetted into 1.5 ml clear microfuge tubes. DNA (approximately 1 µg) was added and the samples illuminated for 5 hrs with occasional shaking at 28°C. The cells were plated in top agar (modified BG-11 plus 0.8% agar) onto BG-11T containing plates (BG-11, 1.5% agar and 5 mM sodium thiosulfate). Plates were illuminated at 28°C for 14 hrs and then subjected to selection by antibiotics. Ampicillin (amp) (final concentration of 1 µg/ml), chloramphenicol (cm) (final concentration of 10 µg/ml) or both were added underneath the agar in 1 ml aliquots. The plates were maintained under constant illumination and the number of resistant colonies was scored after 5 days.

DNA/RNA Isolation

Anacystis cells containing pVAD1 were grown in modified BG-11 medium

supplemented with 1 $\mu\text{g/ml}$ of ampicillin and 10 $\mu\text{g/ml}$ of chloramphenicol. Untransformed cells were grown in the absence of antibiotics. Cells were harvested in late-log phase (3 to 4 days growth) as described above. The final pellet was resuspended in 3 ml of 50/40 (50 mM Tris Cl pH 7.8, 40 mM Na_2EDTA) and ground in liquid nitrogen with a sterilized mortar and pestle. The final fine powder was thawed at 37°C and 10 $\mu\text{g/ml}$ of proteinase K added. After 30 min incubation, the mixture was extracted thrice with phenol/ CHCl_3 (5:1, v/v) and the final aqueous phase was precipitated with ethanol in the presence of 500 mM ammonium acetate. The nucleic acid pellet was resuspended in TE (10 mM Tris Cl pH 7.4, 1 mM Na_2EDTA) and used, without further purification, in DNA and RNA hybridizations.

Nucleic Acid Hybridizations

Restriction enzyme digests of DNA obtained from wild-type and transformed *Anacystis* cells were fractionated on 0.8% agarose gels and transferred to nitrocellulose filters by the method of Southern (23). Hybridization conditions are as described previously (17,24). DNA probes used in DNA and RNA blot analyses were eluted from 1.0% low melting point agarose gels according to the method of Langridge *et al.* (25) and labeled with ^{32}P by nick translation (26,27).

S1-nuclease Mapping

The probe used in these experiments (a 212 bp DNA fragment labeled with ^{32}P at the 5' end of the coding strand) was hybridized to 40 μg of RNA prepared from *Anacystis* cells harboring pVAD1 (described below). The probe extended from a BglII site upstream of the putative promoter region to a PvuII site in the CAT gene. The hybridization reaction (28) was carried out at 48°C for 18 hrs and the products of the S1 nuclease digestion were fractionated electrophoretically on a 6% sequencing gel along-side the reaction products obtained from sequencing the same fragment.

DNA Sequence Analysis

The SmaI-PvuII fragment of pMH1/HS484/4 (described below) containing the ps2B promoter and part of the coding region for the CAT gene was sequenced according to the method of Maxam and Gilbert (29).

RESULTS AND DISCUSSION

Construction of Recombinant Plasmids

As shown in Fig. 1 the *Amaranthus* ps2B conserved promoter-like sequence (found in pAH484) lies within a 162 bp fragment that is flanked by SmaI and HindIII sites. This region was inserted into the plasmid pMH1 at the corre-

Figure 1. Restriction map and nucleotide sequence of the *ps2B* promoter-CAT gene fusion. The putative *ps2B* promoter was obtained from pAH484 (12) as a SmaI - HindIII fragment and joined to the CAT gene as shown in Fig. 2. The characteristic prokaryotic -10 and -35 promoter sequences are underlined (32). Plasmid pAH484 contains the *ps2B* gene obtained from an atrazine resistant biotype of *Amaranthus hybridus*. The amino acid sequences are those for the amino terminus of CAT and agree with published data (33). The SmaI-PvuII fragment of pMH1/HS484/4 (Fig. 2) was sequenced according to the method of Maxam and Gilbert (29). The arrow indicates the *in vivo* (*Anacystis*) transcription start site deduced from S1-nuclease mapping (Fig. 3).

sponding restriction sites (Fig. 2). Plasmid pMH1 (20) contains the coding region of the CAT gene but has the endogenous promoter deleted and a unique HindIII site inserted adjacent to the gene. *E. coli* HB101 cells containing this plasmid (pMH1/HS484/4) are chloramphenicol resistant (data not shown). The linkage of the *ps2B* promoter to the CAT gene was established by sequencing this region of the plasmid (Fig. 1).

In order to introduce this construct into *Anacystis*, pMH1/HS484/4 was fused to a shuttle vector that can replicate in both *Anacystis* and *E. coli*. The plasmid used in these experiments (pUC105-31) is a derivative of pUC105 (amp^r, cm^r) (see ref. 21 for details). It was constructed by deleting 5.0 kb of DNA located between two PvuII sites in pUC105. In pUC105-31, the endogenous CAT gene found in pUC105 is interrupted and a unique blunt-end cloning site (PvuII) is introduced. pUC105-31 and pMH1/HS484/4 were joined as shown in Fig. 2. The resulting plasmid, pVAD1, contains the chloroplast *ps2B* promoter-CAT fusion gene, plasmid replication origins for *E. coli* and *Anacystis* and the amp^r (ampicillin resistance) antibiotic marker. The orientation of plasmid pUC105-31 in pVAD1 was determined and it was shown that the endogenous CAT promoter (still found on pUC105-31) is facing in the opposite direction to that of the CAT gene (data not shown) and therefore should not affect transcription of this gene.

Three control plasmids were constructed containing all of the features of pVAD1 but lacking the inserted chloroplast promoter. Plasmid pMH1/105-31/34 (not shown) is a fusion product of pUC105-31 and pMH1 (see Table 1). In this

Figure 2. Construction of a plasmid containing the chloroplast promoter-CAT fusion, *Anacystis* and *E. coli* plasmid replication origins and amp^r marker. pAH484 contains the *ps2B* gene from *Amaranthus hybridus* (atrazine resistant biotype); pMH1 contains the promoterless CAT gene; and pUC105-31 is a shuttle vector that can replicate in *Anacystis* or *E. coli*. pUC105-31 was obtained by deleting 5.0 kb of DNA located between two PvuII sites in pUC105. The SmaI-HindIII fragment of pAH484 (Fig. 1), containing the putative promoter region of the *ps2B* gene, was inserted into pMH1 at the corresponding sites. The resulting plasmid (pMH1/HS484/4) was fused with pUC105-31 by opening the plasmids at their respective SmaI and PvuII sites and ligating the two blunt-ended fragments together. The resulting plasmid, pVAD1, contains the *ps2B* promoter inserted adjacent to the promoterless CAT gene, *E. coli* and *Anacystis* plasmid replication origins, and the amp^r marker. In pVAD1 the vestigial promoter located on pUC105-31 is facing in the opposite direction to that of the CAT gene.

plasmid, as in pVAD1, the vestigial CAT promoter lies several kilobases away from the CAT gene and is facing in the opposite direction from the gene. The two additional plasmids (pMH1/105ori/19 and pMH1/105ori/42) were constructed utilizing the knowledge that the *Anacystis* plasmid replication origin lies within a BglIII-PvuII fragment of pUC105-31 (V.A.D., G.C.O., and L.B., unpublished results). The construction of these plasmids is described in Table 1.

Introduction, Expression, and Analysis of Recombinant Plasmids in *Anacystis*

The four plasmids described above were introduced into *Anacystis* and the numbers of amp^r , cm^r (chloramphenicol resistance), and amp^r/cm^r colonies were

Table 1. Transformation of *Anacystis* cells with plasmids that contain or lack the chloroplast *ps2B* promoter-CAT gene fusion.

Plasmid	amp ^r	cm ^r	amp ^r /cm ^r
pVAD1	1350	1040	980
pMH1/105-31/34	1440	0	0
pMH1/105ori/19	840	0	0
pMH1/105ori/42	810	0	0
mock transformation (no DNA)	0	0	0

The values indicate the number of amp^r, cm^r, and amp^r/cm^r *Anacystis* colonies obtained per plate after transformation with the plasmid indicated. Transformations were carried out as described in the Materials and Methods. Transformation frequencies are near 1×10^{-6} . (In more recent experiments, 6500 colonies per plate have been obtained with pVAD1). The construction of pVAD1 is shown in Fig. 2; it contains the *ps2B* promoter-CAT fusion. Plasmids pMH1/105-31/34, pMH1/105ori/19, and pMH1/105ori/42 are similar to pVAD1 but lack the inserted promoter sequence. pMH1/105-31/34 is a fusion product of pUC105-31 (Fig. 2) and pMH1; it was obtained by ligation of the plasmids blunt-ended at their respective PvuII and SmaI sites. To construct pMH1/105ori/19, the 2.6 kb BglII-PvuII fragment of pUC105-31 was inserted into the SmaI-BglII sites of pMH1. The plasmid pMH1/105ori/42 was made by inserting the 3.4 kb BamHI-PvuII piece of pUC105-31 between the BglII and SmaI sites of pMH1.

determined (Table 1). Conditions for transformation are given in the Materials and Methods. Cells transformed with pVAD1 yielded amp^r, cm^r and amp^r/cm^r colonies at roughly equal frequencies, while those cells transformed with vectors lacking the chloroplast promoter-CAT fusion gave no cm^r colonies. *Anacystis* cells containing pVAD1 are able to form colonies at chloramphenicol concentrations of up to 40 µg/ml, whereas wild type cells do not survive at 7.5 µg/ml. pVAD1 can be recovered intact from HB101 cells following transformation of the cells with DNA obtained from *Anacystis* cells containing pVAD1 (data not shown). This suggests that the introduced plasmid is replicating autonomously within the transformed cells. However, the possibility that a sub-population of the plasmid may have integrated into the host chromosome is not excluded.

The presence and transcriptional start site of the CAT fusion gene in pVAD1 transformed *Anacystis* cells was established by Southern (23) and S1-nuclease mapping analyses, respectively. The results of these experiments are shown in Figure 3. In a BamHI digest of DNA isolated from *Anacystis* cells transformed with pVAD1, a CAT specific DNA probe hybridizes to a 5.2 kb fragment (panel A, lane 2). An identical sized fragment is seen when the isolated plasmid itself is digested with BamHI and hybridized to the probe (lane 3). Undigested DNA (lane 4) from transformed *Anacystis* cells contains two size populations corresponding to the supercoiled and relaxed forms of the plas-

Figure 3. Panel A. Detection of CAT DNA sequences in pVAD1 transformed *Anacystis* cells. Hybridization of ^{32}P -labeled CAT DNA probe to: BamHI digested wild-type *Anacystis* DNA (lane 1), BamHI digested DNA from *Anacystis* cells transformed with pVAD1 (lane 2), BamHI digested pVAD1 (lane 3), and undigested DNA from *Anacystis* cells harboring pVAD1 (lane 4). The probe used in these experiments is a 1.3 kb EcoRI fragment of pMHI (containing the CAT gene). It was labeled with ^{32}P by nick translation (26,27). DNA digests were fractionated on 0.8% agarose gels and transferred to nitrocellulose filters by the method of Southern (23). Hybridization conditions are as described previously (17,24). Positions of size standards are as indicated. DNA was prepared as described in the Materials and Methods.

Panel B. Results of S1-nuclease protection experiment. A 212 bp DNA fragment labeled with ^{32}P at the 5' end of the coding strand was hybridized to 40 μg of RNA prepared from *Anacystis* cells harboring pVAD1. The probe extended from a BglII site upstream of the putative promoter region to a PvuII site in the CAT gene. The hybridization reaction (28) was carried out at 48°C for 18 hrs and the products of the S1 nuclease digestion (S1 lane) were fractionated on a 6% sequencing gel alongside the reaction products obtained from sequencing the same fragment. The protected fragment aligns the start point of transcription to 8 bp after the consensus -10 box (Fig. -1). Lane 1 shows the products of S1-nuclease digestion following mock hybridization in the absence of RNA.

mid. However, as is discussed above, the possibility that a sub-population of the plasmid may have integrated into the host chromosome has not been excluded. DNA of wild type Anacystis does not hybridize with the probe (lane 1).

The point at which transcription is initiated was established by S1-nuclease analysis (Fig. 3, panel B). The size of the protected fragment indicates that transcription begins 8 base pairs after the consensus -10 box (shown by + in Fig. 1). This site corresponds, within one base pair, to the start sites seen in vivo and in vitro for other plant ps2B genes (Sinapis alba (15), Zea mays (L. Crossland and L. Bogorad, unpublished results)).

Summary

The results reported here indicate that in the cyanobacterium Anacystis nidulans R2 transcription can be accurately initiated from a chloroplast promoter. This finding is consistent with a prokaryotic (possibly cyanobacterial) origin for chloroplast genes. The results also demonstrate the feasibility of using promoterless gene constructions (30,31), such as the CAT gene derivative employed here, for identifying and studying the activity of foreign and endogenous promoter sequences in Anacystis as well as for testing for the ability of chloroplast gene products to function in the cyanobacterial photosynthetic apparatus.

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