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Cloning of a Marine Cyanobacterial Promoter for Foreign Gene Expression Using a Promoter Probe Vector

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ABSTRACT

A marine cyanobacterial promoter was cloned to allow efficient foreign gene expression. This was carried out using chloramphenicol acetyl transferase (CAT) as a marker protein. For rapid and simple measurement of CAT activity, a method based on a fluorescently labeled substrate was improved by utilizing HPLC equipped with a flow-through fluorescent spectrophotometer. This method was used in conjunction with a newly constructed promoter probe vector. Cyanobacterial transformants, harboring plasmid containing a cloned 2-kbp marine cyanobacterial genomic fragment, showed a 10-fold higher CAT activity, compared with that achieved using the kanamycin-resistant gene promoter. From the sequence analysis of the cloned fragment, a putative promoter region was found.

Index Entries: Marine cyanobacteria; promoter; foreign gene expression; fluorescent CAT assay.

INTRODUCTION

Much attention is being paid on the development of bioreactor systems and gene manipulation techniques for marine cyanobacteria, to allow the development of clean bioprocesses based on their photosynthetic activity. Recombinant DNA techniques for marine cyanobacteria may expand

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their potential in the production of useful materials, such as metabolites (*1,2*), cell constituents (*3,4*), and recombinant gene products. In addition, they have potential for solar light energy conversion, for example, hydrogen production (*5*) and, more recently, for biological carbon dioxide fixation systems (*6*).

We have been working on the development of gene manipulation systems for marine cyanobacteria based on transconjugation (*7*). We have used the broad host range vector, pKT230 (*8*) as the plasmid for foreign gene expression. Plasmid stability as well as pseudo-continuous culture were tested to illustrate their potential as suitable host-vector for foreign gene expression (*9,10*).

Chloramphenicol acetyl transferase (CAT) is widely used as an indicator for the measurement of expression efficiency, by using the CAT structural gene fused downstream of an appropriate promoter. With recent advances in the development of nonradioisotopic labeling techniques, a fluorescently labeled chloramphenicol (Bodipy™ chloramphenicol) is extensively utilized. We previously reported CAT expression in marine cyanobacteria under various conditions (*10,11*). However, because conventional procedures for CAT activity measurement using thin-layer chromatography combined with fluorescent spectrophotometer are complicated (*12*), development of a simple system is essential to advance the study of foreign gene expression using CAT as the reporter protein.

Here we report the cloning of a highly active marine cyanobacterial promoter region. To achieve this, we first improved the method for CAT activity measurement based on detection of fluorescently labeled chloramphenicol using HPLC. A promoter probe vector based on pKT230 with promoterless CAT gene was also constructed. A marine cyanobacterial genomic library was cloned into this vector and recombinants expressing high levels of CAT were isolated.

MATERIALS AND METHODS

Chemicals

Restriction enzymes and other DNA modification enzymes were purchased from Toyobo (Osaka, Japan). Fluorescently labeled chloramphenicol, Bodipy™ chloramphenicol was purchased from Molecular Probes (Eugene, OR). Authentic CAT was purchased from Pharmacia (Uppsala, Sweden). All other chemicals used in this study were of analytical grade.

Bacterial Strains and Plasmids

As the host cyanobacterial strain, a marine cyanobacterium, *Synechococcus* sp. NKBG 15041c was used (*7*). The freshwater unicellular cyanobacterial strain, *Anacystis nidulans* R2 (*Synechococcus* sp. PCC 7942), was obtained from the American Type Culture Collection (ATCC). As the donor

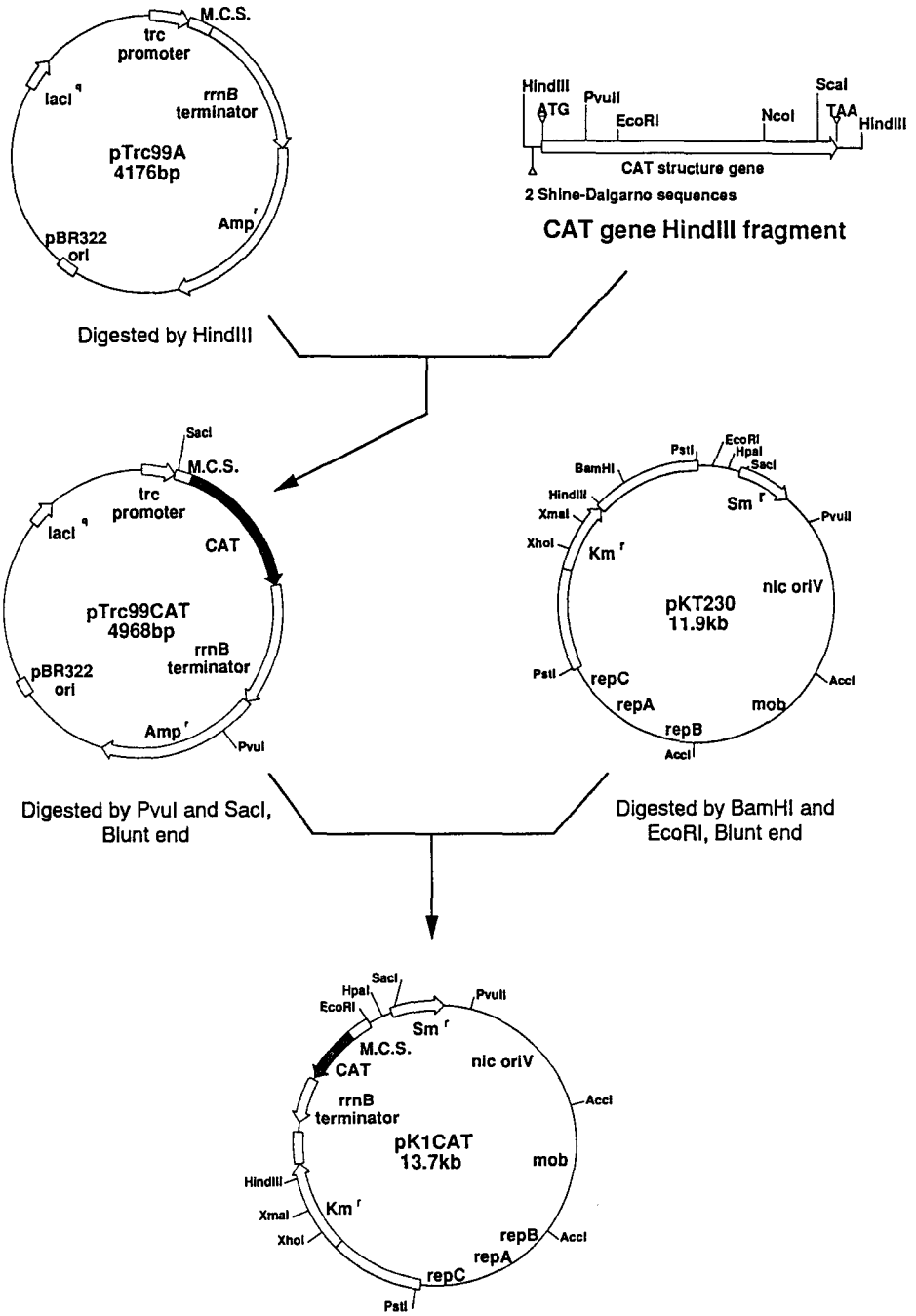

Fig. 1. Construction of promoter probe vector pK1CAT based on broad host range vector pKT230.

strain for transconjugation, *Escherichia coli* S17-1 was used (*13*). A promoter probe vector, pK1CAT, was constructed as follows (Fig. 1). The CAT structural gene, which was obtained as CAT GenBlock (Pharmacia), was digested with *Hind*III and cloned into *Hind*III site of an expression vector, pTrc99A (Pharmacia). From this plasmid, a fragment was obtained by *SacI*

and *PvuI* digestion followed by blunt ending, using T4 DNA polymerase (Takara, Kyoto, Japan). The resulting fragment contained a multicloning site, CAT gene, and terminator (*rrn*B). Plasmid pKT230 was digested with *Bam*HI and *Eco*RI, and ligated with the CAT fragment obtained as above. The constructed plasmid was named as pKICAT (Fig. 1).

Cloning of Marine Cyanobacterial Promoter Regions

Total genomic DNA from NKBG 15041c was extracted according to Porter et al. (*14*), and digested by either *Kpn*I, *Sa*/I or *Xba*I. Restricted genomic DNA was cloned into pKICAT, digested by each corresponding restriction enzyme, and cloned into *E. coli* S17-1. Transformants were selected on the LB agar plates in the presence of 25 μg/mL of kanamycin (Km). Colonies which appeared on the selection plates were picked up and directly used for transconjugation as described previously (*7*). The first selection of marine cyanobacterial transconjugants was carried out in a liquid medium (marine BG11 medium) containing 10 μg/mL of Km. Cells growing in this selection medium were then transferred into media with various concentrations of Km and chloramphenicol (Cm), as shown in Table 1.

Southern Blot and DNA Sequence Analysis

DNA hybridization experiments were carried out using the nonisotopic labeling system [Digoxigenin-11-d uridine triphosphate (UTP), anti-digoxigenin enzyme-linked immunosorbent assay (ELISA)] (Boehringer, Mannheim, Germany). The hybridized digoxigenin-labeled DNA probe was detected using the chemiluminescent substrate, 3-(2′-Spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD^R), according to the manufacturer's instructions.

Cloned DNA fragments from marine cyanobacteria were sequenced using an automated DNA sequencer (Shimadzu DSQ-1, Kyoto, Japan).

Fluorescent CAT Analysis Using HPLC

CAT expression levels were determined using fluorescently-labeled chloramphenicol combined with the improved method. The CAT reaction with fluorescently-labeled chloramphenicol was carried out according to manufacturer's instructions, with a slight modification. The authentic CAT was diluted with 100 m*M* Tris-HCl (pH 8.0). Sixty microliters of the diluted authentic CAT was mixed with 10 μL of Bodipy™ chloramphenicol substrate reagent and incubated at 37°C for 5 min. Ten microliters of 4 m*M* acetyl CoA was added and incubated for a fixed period of time between 1 min and 1 h, depending on the CAT concentration. After incubation, 400 μL of ice-cold ethyl acetate was added. Each sample was vortexed for about 20 s and the samples centrifuged for 3 min to separate the liquid phase. The top 350 μL of ethyl acetate was removed and transferred to a

Table 1
Selection of Transconjugants Harboring pK1CATcyak[a]

Strains	Antibiotics	Concentration (μg/mL)									
		0	5	10	25	50	100	200	400	800	1600
NKBG15041c	Km	+++	−	−	−	−	N.T.[b]	N.T.	N.T.	N.T.	N.T.
	Cm	+++	−	−	−	−	−	−	−	−	
NKBG15041c/pK1CAT	Km	+++	+++	+++	++	+	N.T.	N.T.	N.T.	N.T.	N.T.
(promoterless CAT)	Cm	+++	−	−	−	−	−	−	−	−	
NKBG15041c/pK1CATcyaK	Km	+++	+++	++	+	+	N.T.	N.T.	N.T.	N.T.	N.T.
	Cm	+++	+++	+++	++	++	++	++	+	+	

[a] +, resistant; −, sensitive.
[b] N.T., not tested.

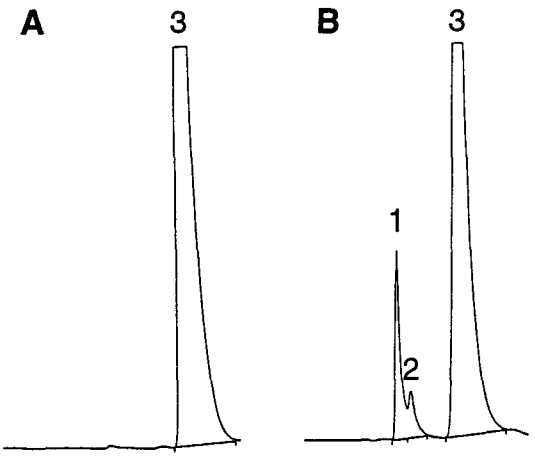

Fig. 2. The chromatogram obtained by HPLC equipped with flowthrough fluorescence spectrometer. (**A**) Bodipy chloramphenicol. (**B**) Bodipy chloramphenicol reacted with extract from CAT produced by a recombinant cyanobacterium for 60 min. Peak numbers 1, 2, and 3 indicate 3-acetyl chloramphenicol, 1-acetyl chloramphenicol and chloramphenicol, respectively.

clean tube. These samples were analyzed by an HPLC system equipped with a flowthrough fluorescent spectrophotometer (Hitachi F-1200, Tokyo, Japan). Twenty microliters of these samples were injected into a Unisil Q100 column (GL Sciences 4.6 × 250 mm, Tokyo, Japan) which was equilibrated with eluent (90% (v/v) chloroform, 10% (v/v) methanol). Then the percent conversion of substrate to acetylated products was obtained from the chromatogram using an integrator (Shimadzu CR-5A, Kyoto, Japan).

The sample for measurement was prepared from culture broth of recombinant marine cyanobacteria harboring pK1CAT containing the cloned promoter region. Cultivation conditions were as described previously (*10*).

RESULTS AND DISCUSSION

Fluorescent CAT Assay Using HPLC

Conversion of chloramphenicol by CAT was determined by monitoring the presence of its acetylated products in the chromatogram obtained by HPLC equipped with a flowthrough fluorescence spectrophotometer (Fig. 2). This chromatogram was obtained within 20 min after sample injection. The conversion ratio, as well as the CAT activity, can be simultaneously determined with the data analysis used for chromatography. When using conventional thin-layer chromatography, at least 30 min is necessary for separation (*15*). Furthermore, it requires the extraction of converted products from each spot on the plate and measurement of each

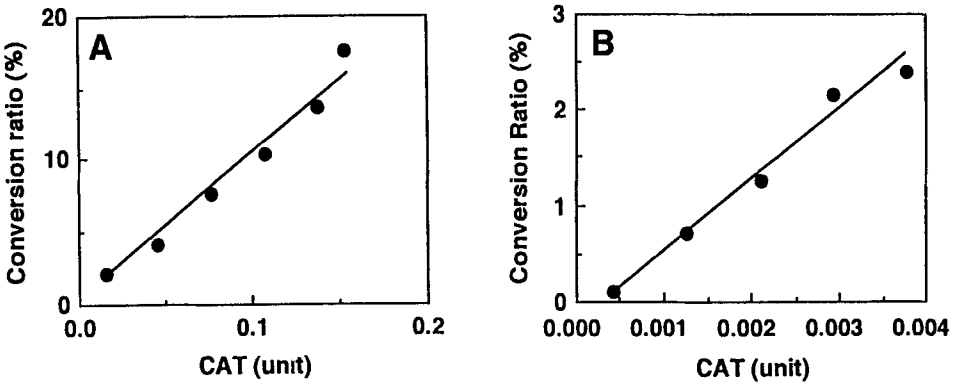

Fig. 3. Correlation between CAT activity and conversion ratio of Bodipy chloramphenicol. Ten nmol of Bodipy chloramphenicol and 40 μmol of acetyl coenzyme A were reacted at 37°C for 1 min (**A**) or 60 min (**B**). Reaction volume is 80 μL.

fluorescence, separately (*12*). Therefore, our method utilizing HPLC reduced time and effort required for CAT analysis.

When 0.7 U of CAT were used for the reaction, conversion was almost complete within 15 min. A good linear correlation between time and conversion ratio was observed within 5 min of incubation. To calculate the CAT activity of the sample, one should carry out the experiment where the conversion ratio is proportional with time. We varied the amount of CAT and time for incubation, and obtained the optimum incubation time to determine the CAT activity (Fig. 3). By making a dilution series of purified CAT with defined activity, the dynamic range and sensitivity of this analyzing system were investigated. Each figure represents the reliability of this method in each range. The highest sensitivity achieved using this method was 4×10^{-4} U, which was similar to that reported using ^{14}C-labeled chloramphenicol as the indicator (*16*). Together with these figures, the wide range of sample concentrations can be measured with high sensitivity.

Cloning of Marine Cyanobacterial Promoter

Using our constructed promoter probe vector, pK1CAT (Fig. 1), the cloning of marine cyanobacterial promoter was carried out. Transconjugants harboring marine cyanobacterial promoters were screened using liquid medium containing various concentrations of Cm and Km to enrich Cm-resistant transconjugants (Table 1). After 10 d of selection, the growth of transconjugants possibly harboring cyanobacterial genomic DNA fragments was observed in the presence of Cm, even at a concentration of 800 μg/mL. In contrast, no growth was observed in host strain and also in the transconjugants not harboring cyanobacterial DNA fragment (pK1CAT). In addition, no difference in the Km resistance was observed between

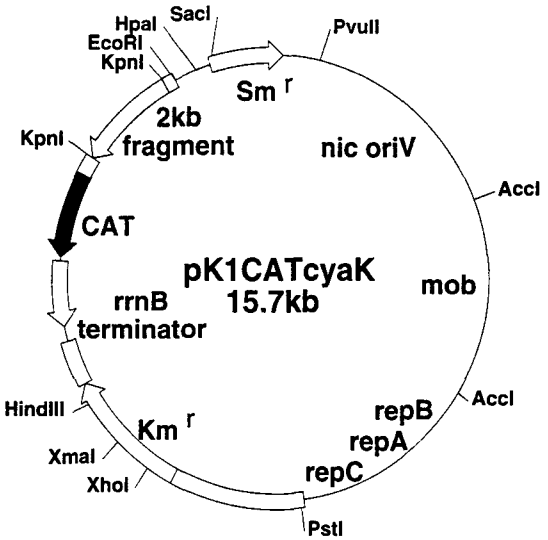

Fig. 4. Structure of plasmid pK1CATcyaK harboring a 2-kbp marine cyanobacterial genomic fragment containing highly active promoter in NKBG 15041c.

pK1CAT and those containing cyanobacterial DNA fragment. Such a high resistance to Cm might be due to the expression of CAT in the marine cyanobacteria. Of the three different libraries, transconjugants harboring promoter probe vectors containing *Kpn*I-digested DNA fragment showed rapid growth. Using this library, we further characterized the marine cyanobacterial promoter. Plasmids were extracted from transconjugants (*14*) and transformed back into *E. coli* DH5α to analyze their restriction pattern (Fig. 4). All plasmids extracted from *E. coli* DH5α harbored a 2-kbp *Kpn*I fragment, indicating that transconjugant expressing high CAT activity contained a similar DNA fragment. Figure 5 shows the results of Southern blot analysis using the 2-kbp *Kpn*I fragment as the probe (Fig. 5A) and agarose gel electrophoresis (Fig. 5B) of *Kpn*I restriction fragments of the genomic DNA of NKBG 15041c, recombinant NKBG 15041c harboring this plasmid, and PCC 7942. Southern blot analysis revealed that this probe hybridized only with 2-kbp *Kpn*I fragments that corresponded to marine cyanobacterial genomic DNA, but not PCC 7942. These results showed that the 2-kbp *Kpn*I fragment was derived from NKBG 15041c, which did not show homology to the PCC 7942 genomic DNA. In further experiments, we designated this plasmid as pK1CATcyaK. We have sequenced the cloned 2-kbp *Kpn*I fragment. Figure 6 shows 240-based nucleotide sequence of cloned *Kpn*I fragment with putative promoter region in its 3′-terminal region.

CAT Expression Using Marine Cyanobacterial Promoter

Figure 7 shows the time-course of CAT expression (Fig. 7A) and cell growth (Fig. 7B) of recombinant marine cyanobacteria harboring

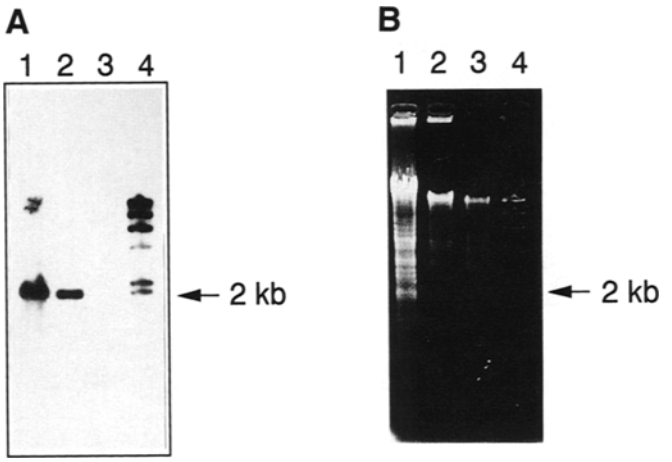

Fig. 5. Southern hybridization analysis of 2-kbp *Kpn*I fragment; (**A**) Southern blot hybridized with 2-kbp *Kpn*I fragment as a probe and (**B**) an agarose gel stained by EtBr. Lanes: 1, total DNA from recombinant NKBG 15041c harboring pK1CATcyaK digested by *Kpn*I; 2, total DNA from NKBG 15041c digested by *Kpn*I; 3, total DNA from PCC 7942 digested by *Kpn*I; 4, digoxigenin-labeled λ-DNA digested by *Hind*III (marker).

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1           11          21          31          41          51
TGGTCCGATG CTANCGACAG GGGGACAATT ATCGCAGCAC TGGCAGCAAC TGACCCAAGC

61          71          81          91          101         111
GGGATCGCCC CAGAGAATGT GCGGATTATC TCGATCGTGG CCGCACCCCC AGCTTTGCAA

121         131         141         151         161         171
AAATTGAGTC AAGACTATCC CACTTTGCAA ATTTATACAG CGATGATTGA CCAAGATCTC
                                                -35
181         191         201         211         221         231
AACGATCAAG GGTTTATTGT GCCGGGCCTG GGGGATGCGG GCGATCGCGC CTTTGGTACC
              -10                                             KpnI
241         251         261         271         281         291
CGGGGATCCT CTAGAGTCGA CCTGCAGGCA TGCAAGCAAG CTTCGACGAG ATTTTCAGGA
                                          HindIII
301         311         321         331         341         351
GCTAAGGAAG CTAAAATGGA GAAAAAAATC ACTGGATATA CCACCGTTGA TATATCCCAA
              →cat
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Fig. 6. Nucleotide sequence of putative promoter region from marine cyanobacterial genomic DNA. Bases 1–239: cloned 3′-terminal genomic DNA from marine cyanobacteria NKBG 15041c. Bases 165–200: putative promoter region including-35 and -10 regions. Bases 316-: CAT structural gene.

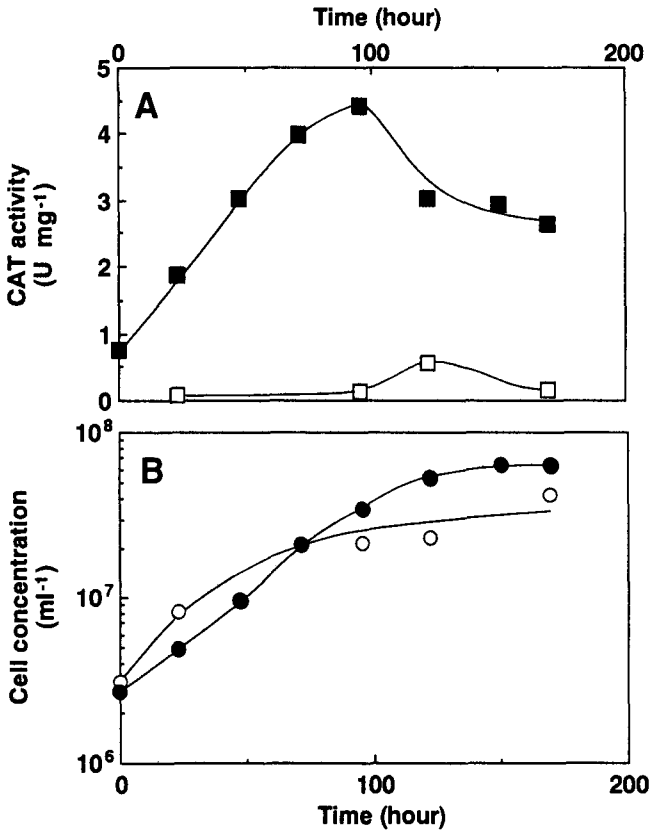

Fig. 7. Time course of CAT activity (**A**) and cell growth (**B**) in a batch culture of recombinant NKBG 15041c harboring pK1CATcyaK (■) and pKCAT2 (□), respectively.

pK1CATcyaK. The CAT activity was determined by the improved fluorescent analysis utilizing HPLC. The cell growth reached stationary phase after 150 h of cultivation. Maximum CAT activity was observed at 100 h, and the level was 10-fold higher than the pKCAT2 recombinant expressing CAT under Km-resistant gene (*Km*^{r}) promoter, which we had previously constructed (*10*). CAT activity gradually increased with cell growth until 100 h, then a decrease in CAT activity was observed. This pattern also had been observed in a culture using pKCAT2 (*10*). Since the cloning vector used in both studies was pKT230, an IncQ broad host range vector, the copy numbers in the same cyanobacterial strain should be similar in both cases. Therefore, the difference in the observed CAT expression level was due to the promoter activity, but not the the copy numbers. pKT230 includes the *oriV* region of plasmid RSF1010, which has copy numbers of 10–12 in *E. coli* (*17*). In cyanobacteria, however, the copy numbers of pKT230 should be lower, since we were able to detect this plasmid from cyanobacteria extract only by using hybridization analysis (*7,9*). In Table 2, the sequence of cloned putative promoter was compared with previously-

Table 2
Comparison of Cloned Putative Promoter Sequence with Other Promoters

Promoter	−35	−10
Kanamycin resistance gene[a]	GATGTTACATTGCACAAGATAAAAATATATCATCAT	
Anabaena psbAI[b]	AGTCTAGTAAATTTGCGTGAATTCATGTAAATTTTAT	
Synechococcus psbAII[c]	GTTCTTTACAAAACTCAATCTGCTTGTTAGATTTTAC	
*Kpn*I fragment [putative]	GATTGACCAAGATCTCAACGATCAAGGGTTTATTGT	

[a]From Tn*903* (*18*).
[b]From *Anabaena* PCC 7120 (*16*).
[c]From *Synechococcus* PCC 7942 (*17*).

reported cyanobacterial promoters (*18,19*) and Km^r promoter (*20*), which was used in pKCAT2. The newly isolated putative promoter sequence from marine cyanobacteria did not show any homology with Km^r promoter, although more than 60 and 50% of homology were observed between *Synechococcus psbA*II and *Anabaena psbA*I promoters, respectively. Although several foreign genes have been expressed in cyanobacteria, little is known about the structure of promoter in cyanobacteria.

In conclusion, we demonstrated the cloning of highly active marine cyanobacterial promoter for foreign gene expression. The elucidation and characterization of detailed structure of the promoter region will allow a higher expression level of foreign gene products.

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