

Thesis for the Degree of Master of Science

Construction of *Chlorella* transformation
vector using promoter and 3' UTR of
Chlorella virus isolated in Korea

by

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February 2002

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한국에서 분리한 클로렐라 바이러스의 프로모터와 3' UTR을
이용한 클로렐라 형질전환 벡터의 구축

Advisor : Tae-Jin Choi

by

Hyoun-Hyang Park

A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Science

in the Department of Microbiology, Graduate School,
Pukyong National University

February 2002

박현향의 이학석사 학위논문을 인준함

2001 년 12 월 26 일

주 심 이학박사 김 영 태 (인)

위 원 농학박사 김 진 상 (인)

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Construction of *Chlorella* transformation vector using promoter and 3' UTR of *Chlorella* virus isolated in Korea

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Abstract

Chlorella is a eukaryotic microalga and shares metabolic pathways with higher plants. These characteristics make *Chlorella* as a potential candidate of eukaryotic overexpression systems. However, the use of *Chlorella* as an expression system has been limited because highly efficient and stable transformation techniques have not been developed. Recently, a foreign gene, the flounder growth hormone gene, was stably introduced and expressed in transformed *Chlorella ellipsoidea* by using a modified plant transformation vector that contains cauliflower mosaic virus (CaMV) 35S promoter and the phleomycin resistant Sh ble gene as a selection marker. In this study, this vector was modified for efficient expression of foreign proteins in transformed *Chlorella*. A promoter for the 33kDa peptide gene was cloned from a *Chlorella* virus isolated in Korea and used to replace the 35S

promoter. In addition, the 3' UTR of the *33kDa* peptide gene was introduced to separate the target gene and downstream Sh ble gene. The human erythropoietin (EPO) gene was cloned into the modified vector. Three different *Chlorella* transformation vectors containing EPO gene were constructed. The mp335EPO vector only consist of a promoter from the *33kDa* peptide gene. The mp3353EPO vector contains a promoter from the *33kDa* peptide gene and 3' UTR. The mp35S33pEPO vector contains a 35S promoter and 3' UTR. Protoplasts prepared from *Chlorella ellipsoidea* were transformed with these vectors and transformants were selected with in the presence of phleomycin. The integration of the introduced DNA was confirmed by PCR amplification of the EPO gene from purified genomic DNA of the transformants. The expression levels of EPO protein from these constructs was analyzed by ELISA method.

Introduction

Chlorella is a unicellular eukaryotic green microalga, which has been widely used for live food in aquaculture and for health food industry. This organism can be cultured inexpensively because it requires only limited amount of minerals and sunlight, and cultured with a large scale in a pool. It also grows relatively fast and divides 2-9 times per day depend on the light intensity and temperature (Sorokin and Krauss, 1958). These advantages make *Chlorella* as a potential candidate of eukaryotic expression systems for the large-scale production of recombinant proteins.

Despite of these advantages, the use of *Chlorella* for the expression of foreign proteins has been limited because of several problems in this system. For example, Jarvis and Brown (1991) reported a transient expression of a firefly luciferase gene in transformed *Chlorella ellipsoidea* but the protein was expressed only for 52 hours. Recently, the flounder growth hormone (fGH) gene was stably introduced and expressed in transformed *C. ellipsoidea* using the modified plant transformation vector (Kim *et al.*, 2001). This vector was constructed with cauliflower mosaic virus (CaMV) 35S promoter for the expression of foreign genes and the phleomycin resistance *Sh ble* gene was used as a selection marker.

Although, it has been reported that CaMV 35S promoter functions in microalgae including *Chlamydomonas* (Dunahay, 1993;

Hasnain *et al.*, 1985), the efficiency of this promoter has not been compared with other promoters originated from microlagae. Recently, the entire genome of a *Chlorella* virus, PBCV-1, has been cloned and sequenced (Gerald *et al.*, 1996). This virus encodes many useful genes including restriction/modification enzymes, topoisomerase, chitinase, hyaluronam synthase (Zhang *et al.*, 1998; Xia *et al.*, 1986; Lavrukhin *et al.*, 2000; Sun *et al.*, 1999; Graves *et al.*, 1999). In addition to these genes, several putative promoter sequences have been identified in the PBCV-1 genome, characterized by high A+T content up to 80% (Anne *et al.*, 1990). One of interesting is the promoter for the 33kDa peptide gene (*33kDa*). The biological function of this protein has not been determined but it is the most abundant protein in the *in vitro* translation of mRNA isolated from PBCV-1 infected cell (Michael and Russel, 1992). This protein was detected at the beginning at 20 min post-infection of virus, which indicated that it is an early protein whose mRNA is transcribed by host transcriptional machinery without the aid of viral transcriptional factors. This is an important factor in search for promoters for the *Chlorella* expression system because this promoter is a strong promoter that has all the necessary information in its own sequence. The *33kDa* gene promoter contains 64% A+T content, which is considerably lower than other promoters identified from PBCV-1 genome (Schuster *et al.* 1990). There were no obvious eukaryotic promoter elements found in this promoter. However, the sequence 5'-AATAAAT, a motif found in the promoters of other PNCV-1

genes, was present approximately 50 bp upstream from the transcription starting point (*tsp*). In addition, the sequence 5'-AAAC was repeated 16 times. This repeated sequences were important for promoter function (Michael and Russel, 1992).

The other factor considered in the vector construction is the termination of transcription. In the vector previously used for *Chlorella* transformation, fGH gene and Sh ble gene could be transcribed as one molecule because there is no sequence for transcription termination of fGH gene (Kim *et al.*, 2001). Schuster *et al.*(1990) reported that the TTTTTNT transcriptional termination motif found from vaccinia virus (Yuen and Moss, 1987) was also present downstream from all 5 major PBCV-1 ORFs. The TTTTTNT motif was present both in the early and late genes of PBCV-1 whereas this sequence is common only for early expressed genes in vaccinia. It is possible that, like vaccinia virus, it signals the termination of transcription for PBCV-1 (Schuster *et al.*, 1990).

In order to determine the promoter activity and the effect of 3' UTR, a gene coding the human erythropoietin (EPO) was used as a target gene. EPO has many functions including stimulation of proliferation, differentiation, and maturation of erythroid progenitors in hematopoietic organs, tissue hypoxia being the major stimulus for erythropoietin production (Jekmann, 1992).

The combinatory effect of *33kDa* promoter and its 3' UTR was analysed using the human EPO gene, and the results were compared to develop a transformation vector for efficient and stable

expression of foreign proteins using transformed *Chlorella*.

Materials and Methods

***Chlorella* transformation vector**

The transformation vector pCTV was constructed by modifying plant transformation vector pMinGFP for expression of fGH (Kim *et al.*, 2001). This vector contains ori V for replication in both *E. coli* and *Agrobacterium*, npt III gene for kanamycin resistance, trf A for replication support, right and left border of T-DNA for integration and Sh ble gene for transformant selection with phleomycin. The fGH gene is under the control of the CaMV 35S promoter and the Sh ble gene is under the control of the RBCS2 promoter of *Chlamydomonas* (Fig. 1).

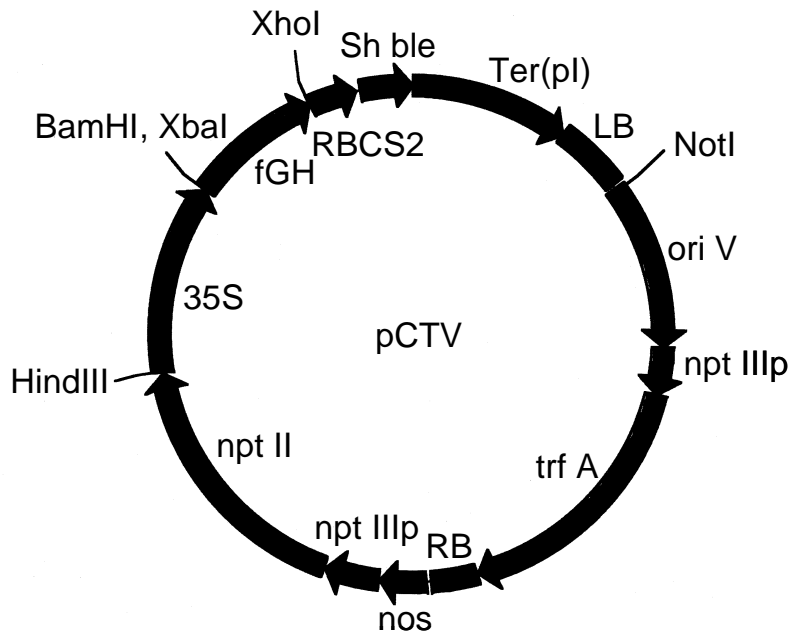


Figure 1. *Chlorella* transformation vector pCTV. In this vector, the fGH gene is under the control of cauliflower mosaic virus 35S promoter and the sh ble gene is under the control of *Chlamydomonas* RBCS2 gene promoter. Ori V, board host range replication origin, RB, right border region; LB, left border region; nos-nptIIIp-npt II, nptII gene under cintrol of nos-npt III double promoter; ter(pl), 3' region of the potato protease inhibitor II gene.

Modification of a *Chlorella* transformation vector

The pCTV vector was designed to introduce any target gene between BamH I and Xho I sites in the vector. However, there is a BamH I site in Sh ble gene that was cloned later in the construction step, which interfere cloning of a target gene (Fig. 2). The BamH I site locates at 21 nt downstream of Sh ble gene stop codon (TAA). There is a Sal I site between the stop codon and BamH I site, and this Sal I site was used to remove the BamH I site. The RBCS2 promoter and Sh ble gene was first amplified with the bleN primer containing a Xho I site and the bleSC primer containing a Sal I site (Fig. 2). The RBC2S-Sh ble fragment was amplified in 30 μ l PCR mixture containing 1 μ l template DNA, 3 μ l of 10 \times buffer (100mM Tris.Cl, pH 8.3, 500mM KCl, 15mM MgCl₂), 0.25mM dNTP (each of ATP, TTP, GTP and CTP), 0.5 μ l Taq DNA polymerase (2.5 μ l/U) and 50 pmole ble-N/ble-SC specific primer (Table 1). The reaction conditions were: 35 cycles of 30 sec denaturation at 95 $^{\circ}$ C, 30 sec annealing at 55 $^{\circ}$ C, 30 sec extension at 72 $^{\circ}$ C followed by 5 min extension at 72 $^{\circ}$ C. The amplified PCR product was analysed on 1% agarose gel electrophoresis after the gel was stained by ethidium bromide. The PCR product was digestion with Xho I and Sal I, and cloned back into pCTV digested with Xho I.

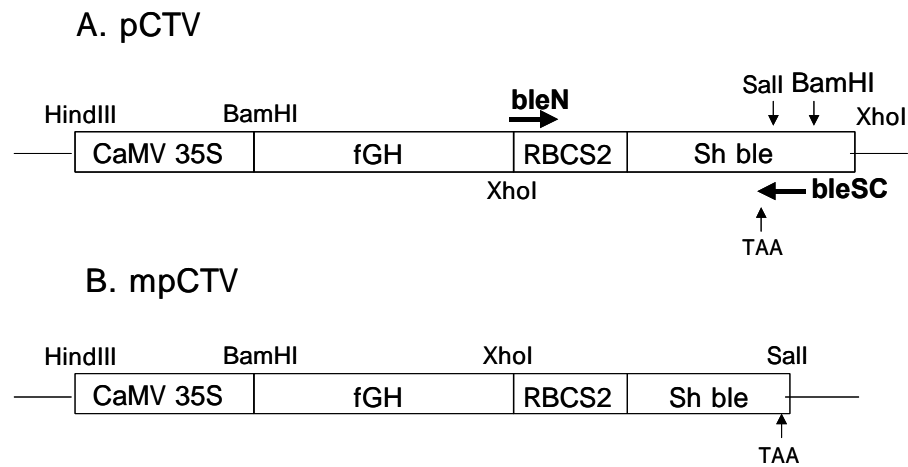


Figure 2. Construction of the mpCTV from pCTV. The pCTV was modified to remove the BamH I site in the Sh ble gene. Sh ble gene was amplified with bleN primer and bleSC primer, digested with Xho I and Sal I, and cloned back into Xho I digested pCTV.

Table 1. Oligonucleotide primers used for subcloning of Sh ble gene.

Oligo	Sequence	Remarks	Description
ble-N	5'-AAACTCGAGGGCGCCAGGAAGGAGC-3'	Xho I	Sh ble gene forward specific primer
ble-SC	5'-GAGTGGGTGAGCGTCCGTTAGTCC-3'	Sal I	Sh ble gene reverse specific primer for midification

Isolation of *33kDa* peptide promoter and 3' UTR from *Chlorella* virus

Twenty three *Chlorella* virus isolates have been isolated from fresh water samples collected from 10 sites in Korea. The *33kDa* gene promoter and 3' UTR were amplified by PCR from 6 isolates, YK-1, YK-2, SS-1, HS, KH and BO. PCR amplification was conducted with virus particles without purification of genomic DNA. Purified virus particles were heated at 98°C for 10 min and 3 μ l of the denatured virus was added to 30 μ l PCR mixture containing 3 μ l of 10 \times buffer (100mM Tris.Cl, pH 8.3, 500mM KCl, 15mM MgCl₂), 0.2mM dNTP (each of ATP, TTP, GTP and CTP), 0.5 μ l Taq DNA polymerase (2.5 μ l/U) and 100 pmole of 335N primer and 335C primer for the promoter and 333N primer and 333C primer for the 3' UTR (Table 2). The reaction conditions were : 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 55°C, 30 sec extension at 72°C followed by 5 min extension at 72°C. The amplified PCR product was analysed on 1% agarose gel and visualized by ethidium bromide staining.

Table 2. Oligonucleotide primers used for subcloning and sequencing of 33kDa promoter and 3'UTR.

Oligo	Sequence	Remarks	Description
335N	5'-GAGGAAGCTTTTGAGACCCTGT -3'	Hind III	33kDa gene promoter forward specific primer
335C	5'-GGCGAATTCGGATCCATGGTATGTTCTTTCTT -3'	BamH I	33kDa gene promoter reverse specific primer
333N	5'-GGCGAATTCCTCGAGATAACTGATTGAATTGAT -3'	Xho I	33kDa gene 3' UTR forward specific primer
333C	5'-TCAGTCGACAGAACATTTCCGTTGGCG -3'	Sal I	33kDa gene 3' UTR reverse specific primer
T7	5'-AATACGACTCACTATAG -3'	-	sequencing primer
T3	5'-ATTAACCCCTCATAAAG -3'	-	sequencing primer
sp6	5'-ATTTAGGTGACACTATAGAATTAC -3'	-	sequencing primer

Cloning and Sequencing of *33kDa* peptide promoter

The *33kDa* peptide promoter amplified by PCR was digested with Hind III and BamH I, and cloned into pBluescript II SK+ vector. The PCR products for the 3' UTR were precipitated with ethanol and cloned into T-easy vector (Promega, USA) for sequence confirmation. The plasmids containing appropriate inserts were purified with plasmid purification kit (Bioneer, Korea) and used as templates for PCR sequencing. Sequencing reaction mixture [promoter : 1000ng template DNA, 4 pmol of T3 primer or T7 primer for the promoter, T7 primer and sp6 primer for the 3' UTR, 2 μ l of terminator ready reaction mix] was prepared, and 25 cycles of PCR reaction composed of 10 sec denaturation at 96°C, 7 sec annealing at 50°C and 4 min extension at 60°C was performed with an Eppendorf Mastercycler 9600 (Germany). The sequencing products were precipitated with ethanol and dissolved in 25 μ l of template suppressing reagent (Perkin Elmer). The DNAs were denatured for 2 min at 98°C and cooled down on ice, and analyzed with a ABI PRISMTM 310 Analyzer (Perkin Elmer).

Isolation of erythropoietin (EPO) gene

A plasmid containing the human EPO gene was kindly provided by Dr. Hyo-Jeong Hong at the Korea Research Institute Bioscience and Biotechnology (KRIBB). The EPO gene was amplified with PCR primers designed for a vector containing the CaMV 35S promoter (pEPON and EPOC primers) and *33kDa* gene peptide promoter (EPON and EPOC primers, Table 3). The PCR was conducted in 30 μ l PCR mixture containing 1 μ l

template, 3 μ l of 10 \times buffer (100mM Tris.Cl, pH 8.3, 500mM KCl, 15mM MgCl₂), 0.2mM dNTP (each of ATP, TTP, GTP and CTP), 0.5 μ l Taq DNA polymerase (2.5 μ l/U) and 100 pmole of each primer. The PCR reaction conditions were : 35 cycles of 30 sec denaturation at 95 $^{\circ}$ C, 30 sec annealing at 55 $^{\circ}$ C, 30 sec extension at 72 $^{\circ}$ C followed by 5 min extension at 72 $^{\circ}$ C. The amplified PCR products were analysed on 1% agarose gel.

Cloning and Sequencing of EPO gene

The EPO gene amplified by PCR was precipitated with ethanol and cloned into pGEM T-Easy vector (Promega) for sequence confirmation. pGEM T-Easy vector plasmid containing EPO gene was purified with plasmid purification kit (Bioneer) and sequenced.

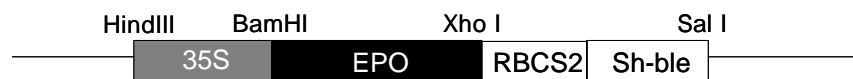
Table 3. Oligonucleotide primers used for subcloning of EPO gene.

Oligo	Sequence	Remarks	Description
EPON	5'-AGATGGATCCGCACGAATGTCCCTGC-3'	BamH I	EPO gene for 33kDa gene promoter forward specific primer
pEPON	5'-CGGGATCCGGTCACTCCCTTATGGGGGTGCACGAA-3'	BamH I	EPO gene for CaMV 35S promoter forward specific primer
EPOC	5'-CCAGCTCGAGACACCCTGTCATCTG-3'	Xho I	EPO gene reverse specific primer
EPOISC	5'-GTGTCAGCAGTGATTGTTCCG-3'	-	EPO gene for detection reverse primer

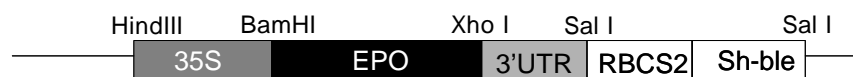
Vector construction containing *33kDa* gene promoter, EPO gene and 3' UTR

In order to compare the effect of the *33kDa* gene promoter and the 3' UTR on the expression of the EPO gene in transformed *Chlorella*, three different expression vectors were constructed as follows. The mp335EPO vector contains a *33kDa* gene promoter and EPO gene, the mp3353EPO vector contains a *33kDa* gene, a EPO gene, and 3'UTR. The *33kDa* gene promoter in the pBluescript SK+ vector was digested with Hind III and BamH I, and cloned into the Hind III and BamH I digested mpCTV to replace the CaMV 35S promoter, resulted in mpCTV335 vector. The EPO gene in pGEM T-Easy vector was digested with BamH I and Xho I, and then cloned into the BamH I and Xho I digested mpCTV335, resulted in mp335EPO vector. The *33kDa* gene 3'UTR in the pGEM T-Easy vector was digested with Xho I and Sal I, and then cloned into Xho I digested mp335EPO, resulting in the mp3353EPO vector. Third vector, mp35S33pEPO that has the CaMV 35S promoter, EPO and the 33kDa gene 3' UTR was constructed by replacing the fGH gene in mpCTV with EPO gene in the pGEM-T easy vector between the BamH I and Xho I site, followed by cloning of the *33kDa* gene 3' UTR downstream of the EPO gene using the Xho I and Sal I sites (Fig. 3).

A. mp335EPO



B. mp3353EPO



C. mp35S33pEPO

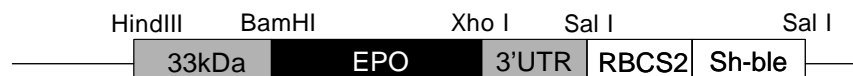


Figure 3. Structure of *Chlorella* transformation vectors constructed in this study.

***Chlorella* Strain and medium**

Chlorella ellipsoidea was provided from Korea Marine Microalgae Culture Center of Pukyong National University (Strain No. KMCC C-20). Cells were cultured in the f/2 medium (Guillard and Ryther, 1962) containing 50 μ g/ml each of chloramphenicol and streptomycin without shaking. Cells were inoculated at initial concentration of 1×10^6 cells/ml and cultured under 3000 lux fluorescent lamp at 25°C with 18:6 h light:dark cycles.

Protoplast preparation

The cells were harvested for protoplast formation when cells reach $1-2 \times 10^8$ cells/ml, usually 8-9 days after inoculation. *Chlorella* cells in 50ml of above culture were harvested by centrifuging for 5 min at 3,000 \times g. Cells were washed once with 25mM phosphate buffer (pH 6.0) and suspended in 5ml of same buffer containing 0.6M sorbitol, 0.6M mannitol, 4%(w/v) cellulase (Calbiochem), 2%(w/v) Macerase (Calbiochem), and 1% (w/v) pectinase (Sigma). The cell suspension was incubated at 25°C for 16 h in the dark with gentle shaking.

***Chlorella* transformation**

Protoplasts were centrifuged at 400 \times g for 5 min and the supernatant was decanted. Protoplasts were gently suspended with 5ml of f/2

medium containing 0.6M each of sorbitol and mannitol, and washed by centrifugation at $400 \times g$ for 5 min. The pellet was suspended in 1ml 0.6M sorbitol/mannitol with 0.05M CaCl_2 . Protoplasts in 0.4ml ($10^7 \sim 10^8$ cells) were placed into new microcentrifuge tube and 5 μg of vector DNA was added with 25 μg calf thymus DNA as carrier (Sigma). After 15 min incubation at room temperature, 200 μl of PNC [0.8M NaCl, 0.05M CaCl_2 , 40% PEG 4000 (Sigma)] was added with gentle mixing. After 30 min incubation at room temperature, 0.6ml of f/2 medium containing 0.6M sorbitol/mannitol, 1% yeast extract and 1% glucose was added, and the cells were incubated at 25°C for 12h in dark. The transformed cells were transferred to fresh f/2 containing 1 $\mu\text{g}/\text{ml}$ of phleomycin and cultured under 3000 lux fluorescent lamp at 25°C with 18:6 h light:dark cycle.

Selection of transformants

Chlorella was transformed with the constructed vectors and selected with phleomycin. The transformants were transferred regenerated cell wall to f/2 medium containing 1 $\mu\text{g}/\text{ml}$ of phleomycin and cultured under 3,000 lux fluorescent lamp at 25°C with 18:6 h light:dark cycle. After 8 days culture, cells were transferred to fresh medium containing same concentration phleomycin.

Isolation of *Chlorella* genomic DNA

Genomic DNA from *Chlorella* was isolated as described by Dawson *et al* (1997). Three milliliters of cells grown to approximately 1×10^8 cells/ml were pelleted and resuspended in 500 μl of CTAB buffer [250 ml: 5 g of hexadecyltrimethylammonium bromide (CTAB), 25 ml of 1 M Tris (pH 8.0), 20.45 g NaCl, 1.68 g EDTA and 10 μl of β -mercaptoethanol (2%)]. The mixture was incubated at 65°C for 1 h and then extracted with equal volume of phenol/chloroform. The extract was centrifuged at $3,000 \times g$ for 5 min and aqueous phase was transferred to a new tube. Extraction was repeated until aqueous layer was no more cloudy. The DNA was precipitated with 0.7 volume of 100% ethanol and pelleted for 15 min at $1,7000 \times g$. The pellet was washed with 70% ethanol and resuspended in 30 μl TE buffer.

PCR of EPO gene from transformed *Chlorella* genomic DNA

The PCR primer pairs, EPON/EPOC and pEPON/EPOC were used to amplify EPO gene in the pGEM T-easy vector, respectively. Another primer, EPOISC (Table 3), located in EPO gene ORF was used to amplify part of EPO gene in combination with EPON primer. One microliter genomic DNA and 100 pmole of each primers were used for reaction in 30 μl and the reaction conditions were: 30 cycles of 1 min denaturation at 94°C, 30 sec annealing at 55°C for *33kDa* promoter and EPO gene, 54°C for 33kDa gene 3' UTR, and 1 min extension at 72°C followed by 5 min extension at 72°C. The amplified PCR product was analysed on 1.5% agarose gel.

Probe preparation

Templates for EPO gene probe synthesis were prepared by PCR using the sequence confirmed above. The probe was synthesized using the DIG (digoxigenin) oligonucleotide 3'-end labeling kit (Boehringer Mannheim, Germany). The template was denatured by heating for 10 min in a boiling water bath and quickly chilled on ice, mixed with $2\mu\ell$ of hexanucleotide mix, $2\mu\ell$ of dNTP mixture and $1\mu\ell$ Klenow enzyme, and then incubated for 18 h at 37°C . The quality of the probe was immunologically determined using the DIG-DNA Detection kit (Boehringer Mannheim).

Southern blot analysis

The PCR products of EPO gene from purified genomic DNA was electrophoresed on 1.5% agarose gel. The gel was transferred to 0.2N HCl and was incubated for 15 min with gentle agitation. The gel was then transferred to denaturation solution (0.5N NaOH, 1.5N NaCl) and incubated for 45 min with gentle agitation. The gel was washed with distilled water for 10 sec and incubated in neutralization solution (0.5M Tris-Cl, pH 8.0, 1.5M NaCl) for 30 min. The DNA was transferred onto nitrocellulose (NC) membrane for 10 h using capillary transfer method, and cross-linked by using a UV cross-linker (Hoefer) with preset condition ($1200\ \mu\text{J}/\text{cm}^2$ at 254 nm). The NC membrane was prehybridized with standard hybridization buffer ($5\times\text{SSC}$, 0.1% N-laurolsarcosine, 0.02% SDS, 1% blocking reagent). Dig-labelled probe was heat-denatured in boiling water bath for 10 min and added to NC membrane. Hybridization was performed at 68°C for 16 h in the same solution. The membrane was washed twice with $2\times\text{SSC}$, 0.1%

SDS at 68°C for 15 min.

After hybridization and stringent washes, the membrane was rinsed in maleic acid buffer (0.1M maleic acid, 0.15M NaCl, pH 7.5) and blocked with blocking buffer (1/10 volume of 10% blocking buffer into maleic acid) for 30 min at room temperature. Anti-Dig antibody conjugated was diluted to 75 mU/ml (1:10,000) in blocking solution for 30 min. After this incubation, NC membrane was washed twice for 15min in maleic acid buffer. The membrane was equilibrated in detection buffer (0.1M tris-Cl, pH 9.5, 0.1M NaCl, 50mM MgCl₂) for 2 min. Then, 45 μ l of NBT and 35 μ l of BCIP were added to 10ml of detection buffer and the membrane was developed in the dark.

ELISA

EPO protein expressed in transformed *Chlorella* was quantified by Enzyme-Linked Immunosorbent Assay (ELISA). Ten milliliter of transformed *Chlorella* (1×10^8 cells/ml) were centrifuged for 10 min at 2,000 \times g. The pellet was immersed in liquid nitrogen and crushed with a homogenator (Wheaton) and centrifuged for 1 min at 13,000 \times g. The supernatant was diluted 1:10, 1:20 and 1:50 in 100 μ l of phosphate-buffered saline buffer (PBS; 135mM NaCl, 2.7mM KCl, 8mM NaHPO₄, 1.5mM KH₂PO₄, pH 7.4). A 96 well assay plate (CORNING, USA) was coated with 100 μ l sample solution at 37°C for 2h. The plate was washed three times with PBS containing 0.08% Tween-20 (PBS-T). Then, blocking buffer (PBS-T plus 2% bovine serum albumin) was added into each well. The plate was incubated for 30 min at 37°C. After removal of blocking buffer, antibody against EPO from

rabbit (diluted 1:1,000 in PBS-T) was added and the plate was incubated at 37°C for 2h, washed, and blocked as described above. Alkaline phosphatase conjugated anti-rabbit IgG serum diluted 1:30,000 in PBS-T was added to each well and incubated at 37°C for 2h. The plate was washed three times with PBS-T, followed by one wash with distilled water. Substrate (10mg p-nitrophenyl phosphate dissolved in 10ml of Diethanolamine buffer, pH 9.8) was added to each well, and the plate was incubated at room temperature for 30min and the reaction was stopped with 100 μ l of 0.1M EDTA. The optical density (OD₄₀₅) was measured at 405nm with UV 900C (Bio-Tek instruments Inc.).

Results

Isolation of *Chlorella* virus *33kDa* gene promoter

Among 6 *Chlorella* virus isolates used for PCR amplification of the *33kDa* gene promoter, PCR products were detected from YK-1, YK-2, SS-1, HS, KH and BO (Fig. 4). However, the size of PCR products was vary, approximately 410bp in YK-1, 480bp in YK-2, 400bp in KH and 580bp in BO. There was no PCR product detected from the isolates SS-2 and HS (Fig. 3). The *33kDa* gene promoter of YK-1 isolate was cloned into pBluescript SK+ vector digested with Hind III and BamH I. Sequencing of the clone showed an insert of 405bp. This sequence was compared with the sequence of the *33kDa* gene promoter of the prototype *Chlorella* virus strain, PBCV-1, and two viruses showed 96% nucleotide sequence homology in this region.

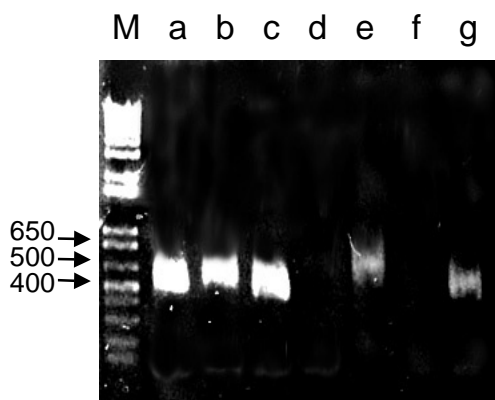


Figure 4. PCR amplification of the *33kDa* gene promoter from *Chlorella* virus isolated in Korea. The PCR product YK-1 isolate was cloned into pGEM T-Easy vector for sequencing, and of used for the construction of modified *Chlorella* transformation vector. Lane M, 1kb plus DNA size marker; lane a, YK-1; lane b, YK-2; lane c, KH; lane d, SS-2; lane e, BO; lane f, HS; lane g, PBCV-1.

PBCV-1 a agcttttgag acctgtttta tataacttcg gtgtggtagg aaaacggggt ccagtaccg
 YK-1 a agcttttgag acctgtttta tataacttcg gtgtggtagg aaaacggggt ccagtaccg

gttgggtggt gccggcgacg atagcatccg tggttttggt ggttcttacc ttgattacct tgatcaaaat
 gttgggtggt gccggcgacg atagcatcag tggttttggt ggttcttacc ttgattacct tgatcaaaat

ttacgagagt atcaccactg gaaagtgcag gatgtgtggg acaatacata aaaatgacaa atgacaaata
 ttacgagagt atcaccactg gaaagtgcag ggtatgtggg gtgaaacata aaa-tgacaa atgacaaata

aatgacaaat aaatgacaac catcatatcg acaagaaaaa gcatttaagc aactgttttc acactaaact
 aatgacaa-- -----c cgtcatatcg acaagaaaaa gcatttaagc aactgttttc acactaaact

catcttcctc cttcgggact accatctgaa caactactga acaacaacac aaacaacaa acaacaacac
 catcttcctc cttcgggact accatctgaa caactactga acaacaacac aaacaacaa acaacaacac

aaacaacaa acaacaacac gctctccgta aacaaacctc aataaccctc aaacaactc cc-aaagaaa
aaacaacaa--- -----c gctctccgta aacaaacctc aataaccctc aaacaactc cccaaagaaa

agaacata--atgg
 agaacataccatggatcc

Figure 5. Comparison of the 33kDa promoter sequence of PBCV-1 and YK-1. The repeated 5' -AAAC sequences are underlined.

Cloning and Sequencing of the 3'UTR of the *33kDa* gene

The 3' UTR of the *33kDa* gene was amplified from 6 *Chlorella* virus isolates. Although PCR products were detected from all of the isolates, the size of them was vary, which was approximately 600bp in SS-1, 490bp in HS, 500bp in YK-1, 520bp in YK-2, 600bp in KH and 490bp in BO (Fig. 6). The PCR product from YK-1 isolate was cloned into pGEM T-Easy vector and its sequence was determined. Sequence analysis of the 3' UTR of the *33kDa* gene showed 88% nucleotide sequence homology to the 3'UTR of PBCV-1 *33kDa* gene (Fig. 7). The sequence of TTTTTTTT, that is known to be important termination in vaccinia virus early genes (Yuen and Moss, 1987), was not found in the 3' UTR of the *33kDa* gene of YK-1 isolate.

Isolation of erythropoietin (EPO) gene

EPO gene for the *33kDa* gene promoter was amplified using the pairs of EPON and EPOC primers, and cloned into pGEM T-Easy vector. The sequence analysis of the cloned gene showed that this is an authentic human EPO gene (Fig. 8). This clone was used in the construction of modified *Chlorella* transformation vector.

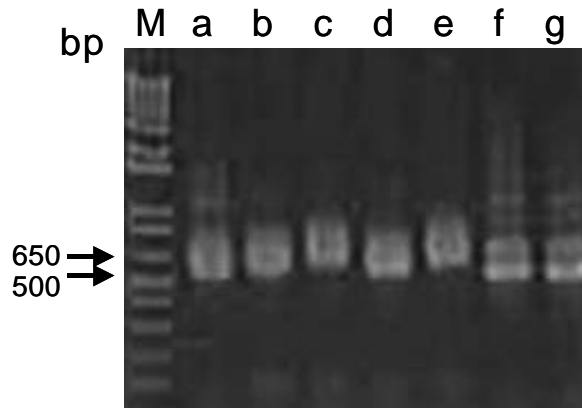


Figure 6. PCR application of the 3' UTR of *33kDa* gene from *Chlorella* virus isolated in Korea. PCR product from YK-1 isolate was cloned into pGEM T-easy vector for sequencing and constructed into mpCTV. Lane M, 1kb plus DNA ladder size marker (Gibco BRL); lane a, amplified PBCV-1; lane b, amplified YK-1; lane c, amplified YK-2; lane d, amplified KH; lane e, amplified SS-2; lane f, amplified BO; lane g, amplified HS.

PBCV-1 cctttgataa ctgattgaat tgattgaaat g-----t aaaaatt-at
 YK-1 ctcgagataa ctgattgaat tgattgaaat gattcgaaat gatgaaatgt aaaaatccac
 * * * * * *
gtaaaaata taa-ttatac ttataaatgg tgg-aaacaa cgcaacactt c-gtatcaat
 gtaaaaata taaattatac ttataaacgg tagcaataa cacaacactt cagtatcaat

 c-gagagctc aaacagacca gaccggcaa acacgactcc ggccaattat agcatacaat
 ctgagagttc aaacagacca ggcccggcaa acacgaactc gacaggttat agcatacaat

 taccacagag atacagaaac atatggtcag ctatgt--tg gtgaatatag cacttccggc
 taccacagag atacaggagc gcatatggag cgctatggtg gt-aatatag cacttccggc

 agtgagccct cctcaaaaat atgtctatct agacatagac aaactcaatt caatcgattc
 agtgagccct cctcaaaaat atgtctatct agacatagac aaactcaact caatcgattc

 tacttctcca tcaggtggg tcaattttgc acttgcaaaa atccctttgt ctatagcggg
 cacttctccg tcagggggg tcaattttgc acttgcaaaa atccctctgt ctatagcggg

 gaccggtaat **gttttttt**g cggacacgat gacttcttct tt-ccccaac gttccgttac
 tacgggtaat gtatattttg cggacacgat gacttcttct ttgcc-tagc gttccgttac

 aaaatccagt tgcaacgatg gataaa-ctt aatataaaac tgaaagacgc caacggaaat
 aaaaccgggt tgcaacanat ggataaactt aatataaaac tgaaagacgc caacggaaat

 gttctgacaat
 gttctgtcgac

Fig. 7. Comparison of the 3' UTR sequence 33kDa gene of PBCV-1 and YK-1. The repeated sequences of 5'-TGATTGAA and 5'-ATGTAAAAA are underlined. The sequence of TTTTTTTT for transcript termination is in bold. The asterisk indicates stop codon, TGA.

1 CCCGGAGCCGGACCGGGGCCACCGCGCCCGCTGCTCCGACACCGCGCCCTGGACAG
 61 CCGCCCTCTCCTCCAGGCCCGTGGGGCTGGCCCTGCACCGCCGAGCTTCCCGGGATGAGG
 121 GCGCCCGGTGTGGTCACCGGGCGGCCAGGTCGCTGAGGGACCGCGCCAGGCGCGGA
 181 GATGGGGGTGCACGAATGCTCTGCCTGGCTGTGGCTTCTCTGTCCCTGCTGTCGCTCCC
M G V H E C P A W L W L L L S L L S L P
 241 TCTGGGCTCCCAGTCTGGGCGCCCACCACGCCTCATCTGTGACAGCCGAGTCTGGA
 L G L P V L G A P P R L I C D S R V L E
 301 GAGGTACCTCTTGGAGGCCAAGGAGGCCGAGAATATCACGACGGGCTGTGCTGAACACTG
 R Y L L E A K E A E N I T T G C A E H C
 361 CAGCTTGAATGAGAATATCACTGTCCCAGACACAAAGTTAATTTCTATGCCTGGAAGAG
 S L N E N I T V P D T K V N F Y A W K R
 421 GATGGAGGTCGGGCGAGGCCGTAGAAGTCTGGCAGGGCTGGCCCTGCTGTCGGAAGC
 M E V G Q Q A V E V W Q G L A L L S E A
 481 TGTCTGCGGGGCCAGGCCCTGTTGGTCAACTCTTCCAGCCGTGGGAGCCCTGCAGCT
 V L R G Q A L L V N S S Q P W E P L Q L
 541 GCATGTGGATAAAGCCGTCAAGTGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCTCGG
 H V D K A V S G L R S L T T L L R A L R
 601 AGCCGAGAAGGAAGCCATCTCCCCTCCAGATGCGGCCTCAGCTGCTCCACTCCGAACAAT
 A Q K E A I S P P D A A S A A P L R T I
 661 CACTGCTGACACTTTCGCAAACTCTTCGAGTCTACTCCAATTTCTCCGGGAAAGCT
 T A D T F R K L F R V Y S N F L R G K L
 721 GAAGCTGTACACAGGGGAGGCCTGCAGGACAGGGGACAGATGACCAGGTGTGTCCACCTG
K L Y T G E A C R T G D R *
 781 GGCATATCCACCACCTCCCTCACCAACATTGCTTGTGCCACACCCTCCCCGCCACTCCT
 841 GAACCCCGTCGAGGGGCTCTCAGCTCAGCGCCAGCCTGTCCATGGACTCCAGTGCCA
 901 GCAATGACATCTCAGGGGCCAGAGAACTGTCCAGAGCAACTCTGAGATCTAAGGATG
 961 TCACAGGGCCAACTTGAGGGCCAGAGCAGAAAGCATTTCAGAGAGCAGCTTTAAACTCAG
 1021 GGACAGAGCCATGCTGGGAAGACGCCTGAGCTCACTCGGCACCCTGCAAAAATTTGATGCC
 1081 AGGACACGCTTTGGAGGCGATTTACCTGTTTTGCGACCTACCATCAGGGACAGGATGACC
 1141 TGGAGAAGTGTAGGTGGCAAGCTGTGACTTCTCCAGGTCTCACGGGATGGGCACTCCCTT
 1201 GGTGGCAAGAGCCCCCTTGACACCGGGGTGGTGGGAACCATGAAGACAGGATGGGGGCTG
 1261 GCCTCTGGCTCTCATGGGTCCAAGTTTTGTGATTCTTCAACCTCATTGACAAGAAGCTG
 1321 AAACCACCAAAAAAAAAAAAAA

Figure 8. The nucleotide sequence of human EPO gene. The location of primers for PCR amplification were underlined and used for vector construction and modification.

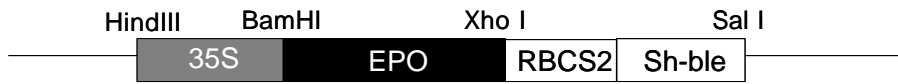
Construction of mp335EPO vector

The *33kDa* gene promoter in pBluescript SK+ vector was digested with Hind III and BamH I and cloned into mpCTV digested with same enzymes to replace the CaMV 35S promoter. Cloning of EPO gene from pGEM T-Easy vector into this structure between BamH I and Xho I site resulted in *Chlorella* transformation vector mp335EPO (Fig. 9).

Construction of mp3353EPO vector

The 3'UTR *33kDa* gene of YK-1 in T-easy vector was digested with Xho I and Sal I, and then cloned into Xho I digested mp335EPO. The resulted clone was first tested for the presence of the insert by PCR amplification of the 3' UTR with 333N and 333C primers, and then the orientation of insert was confirmed by digestion of plasmid with Xho I. The plasmid containing the 3' UTR in correct orientation was selected and named as mp3353EPO vector.

A.



B.

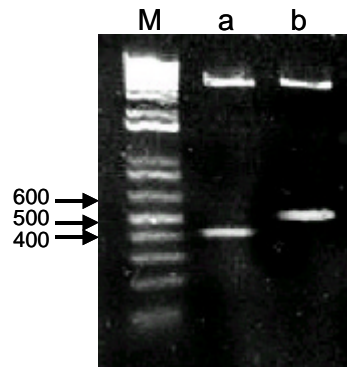
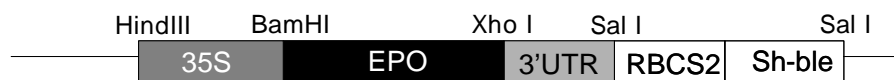


Figure 9. Structure of *Chlorella* transformation vector mp335EPO. The arrangement of the promoter, EPO gene and Sh ble gene is depicted in panel **A** and the confirmation of structure is shown in panel **B**. Lane M, 100bp DNA ladder size marker; lane a, Hind III/BamH I digestion; lane b, BamH I/Xho I digestion.

A.



B.

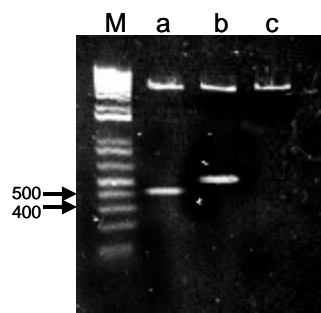


Figure 10. Structure of *Chlorella* transformation vector mp3353EPO.

The arrangement of the promoter, EPO gene and Sh ble gene is depicted in panel **A** and the confirmation of structure is shown in panel **B**. Lane M, 100bp DNA ladder size marker; lane a, Hind III/BamH I digestion; lane b, BamH I/Xho I digestion.

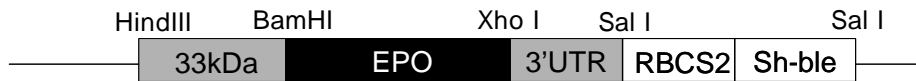
Construction of mp35S33pEPO vector

Plasmid mp35S33pEPO that contains EPO gene under the control of CaMV 35S promoter was constructed to compare the efficiency of *Chlorella* virus *33kDa* gene promoter and CaMV 35S promoter. First, the 3' UTR of *33kDa* gene was cloned between Xho I and Sal I site of mpCTV vector. Subsequence cloning of EPO gene in pGEM T-Easy vector into BamH I and Xho I site resulted in transformation vector mp35S33pEPO (Fig. 11).

Transformation of *Chlorella* cells

Chlorella protoplasts were prepared and transformed with three vectors as described. Cell wall was regenerated from protoplasts by incubating transformed protoplast overnight in the presence of osmotic stabilizer. Transformed cells were then selected by adding phleomycin at the final concentration of $1\mu\text{g}/\mu\text{l}$. The initial cell number was 3.0×10^7 cells in 40 ml media, and cell growth was detected after 7 days incubation. The cell count reached 10^8 cells /ml in 10 days. The cells were transferred into fresh f/2 medium containing $1\mu\text{g}/\mu\text{l}$ phleomycin at a 10 days intervals. There was no growth of non-transformed cell in the presence of $1\mu\text{g}/\mu\text{l}$ phleomycin and green color disappeared after 7 days (Fig. 12).

A.



B.

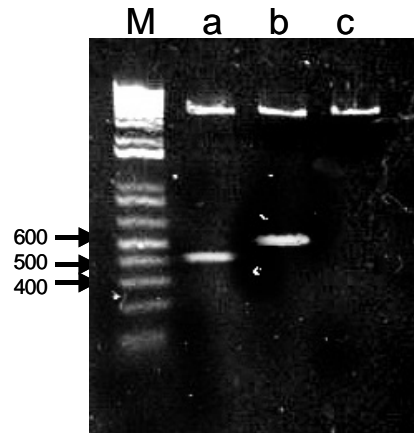


Figure 11. Structure of *Chlorella* transformation vector mp3353EPO.

The arrangement of the promoter, EPO gene and Sh ble gene is depicted in panel **A** and the confirmation of structure is shown in panel **B**. Lane M, 1kb plus DNA size marker; lane a, HindIII/BamH I; lane b, BamHI/Xho I; lane c, XhoI/Sal I.

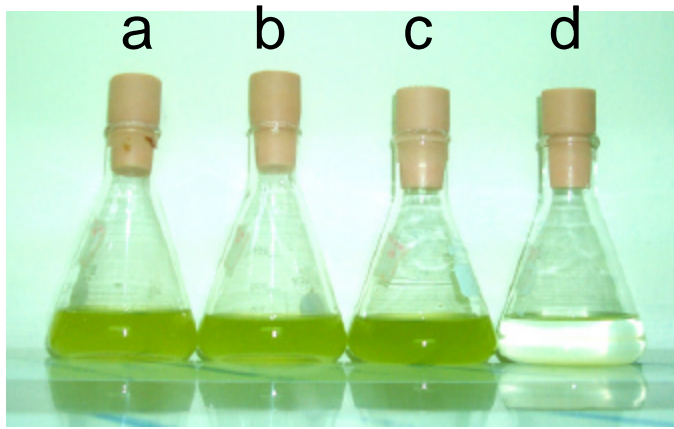
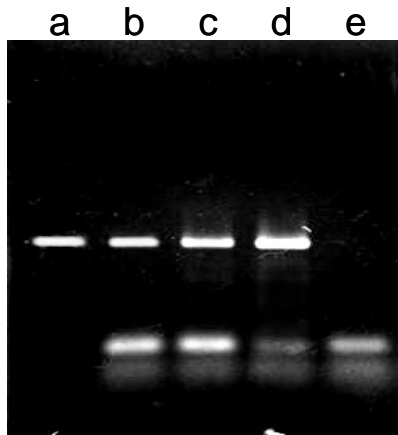


Figure 12. Selection of transformed *Chlorella*. The cells of transformed and non-transformed *Chlorella* were cultured in the presence of $1\mu\text{g}/\mu\text{l}$ phleomycin. a, *Chlorella* transformed with mp35S33pEPO vector; b, *Chlorella* transformed with mp335EPO vector; c, *Chlorella* transformed with mp3353EPO vector; d, non-transformed *Chlorella*. The green color of non-transformed *Chlorella* is disappeared after 7 days.

Detection of the EPO gene from transformed *Chlorella*

Stable integration of introduced DNA into genomic DNA is prerequisite for usage of this transformed *Chlorella* as a bioreactor. The integration of introduced DNA into *Chlorella* genomic DNA was tested by PCR using isolated genomic DNA. The genomic DNA was isolated from the cells of transformed and untransformed *Chlorella* and used as template for PCR amplification of EPO gene. EPO gene was first amplified with the EPON and EPOC primers. PCR products of expected size were detected from all of *Chlorella* cells transformed with mp335EPO, mp3353EPO and mp35S33pEPO vector. However, a PCR product of the same size was also detected from non-transformed *Chlorella* cells. Because wild type *Chlorella* did not grow in f/2 medium containing $1\mu\text{g}/\mu\ell$ phleomycin, it was concluded that this PCR product was produced by non-specific amplification. This was confirmed by designing another PCR primer EPOISC. PCR amplification of EPO gene with the pair of EPON and EPOISC primers produced DNA fragment of the expected size from only the *Chlorella* cells transformed with mp335EPO, mp3353EPO and mp35S33pEPO vectors but not from non-transformed *Chlorella* cells (Fig. 13). The PCR product was further confirmed by Southern blot analysis with EPO gene probe, which showed specific hybridization of the probe to PCR products from transformed cells.

A.



B.

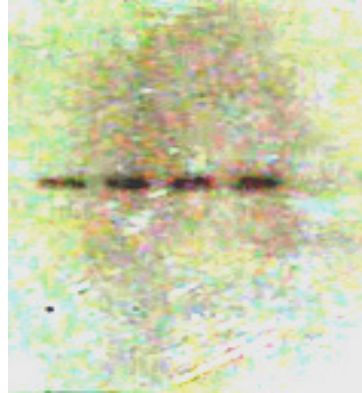


Figure 13. PCR amplification of EPO gene from genomic DNA isolated from various transformation vectors (A) *and* southern blot analysis with EPO gene probe (B). Lane a, positive control; lane b, mp35S33pEPO; lane c, mp335EPO; lane d, mp3353EPO; lane e, wild type.

Expression of erythropoietin protein in *Chlorella*

The expression of introduced EPO gene was tested by ELISA analysis. Total proteins from transformed and non-transformed *Chlorella* were analyzed by the usage of antibody against EPO protein. EPO protein was detected only from transformed *Chlorella* but not from non-transformed negative control (Fig. 14).

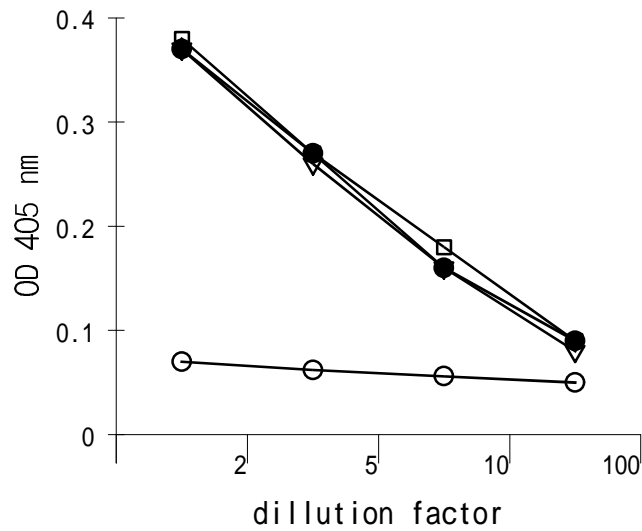


Figure 14. Detection of the expressed EPO protein from transformed *Chlorella* and non-transformed *Chlorella*. White circle, non-transformed *Chlorella*; black circle, *Chlorella* transformed with mp3353EPO vector; white square, *Chlorella* transformed with mp335EPO vector; white triangle, *Chlorella* transformed with mp35S33pEPO vector.

Discussion

Although *Chlorella* has many attractive characteristics required for a good heterologous expression system, development of this organism as an expression system has been hampered by the lacks of appropriate transformation vector. Recent achievement of *Chlorella* transformation and expression of a foreign gene using the flounder growth hormone gene, suggested that foreign genes can be stably integrated and expressed in transformed *Chlorella* (Kim *et al.*, 2001). In addition, it was proved that the expressed protein has biological activity and promoted the growth of juvenile flounder when the protein was indirectly provided to small fish through zooplanktons fed on the transformed *Chlorella*.

Although the CaMV 35S promoter has been used in the transformation of microalgae and has been proven to work in them (Amitava *et al.*, 1994; Eric *et al.*, 1991; Lichard and Michi, 1999; Chow and Tung, 1999; Ying *et al.*, 2001), promoters derived from *Chlorella* would be suitable to use the *Chlorella* expression for overexpression of proteins of interest. It has been shown that a promoter isolated from the typical *Chlorella* virus, PBCV-1, could work in plant (Amitava *et al.*, 1994). Although PBCV-1 encodes a number of transcriptional factors in its 340kb genome (Ann *et al.*, 1990), it is believed that this virus uses host transcription machinery to make its transcripts (Schuster *et al.*, 1986a; Van Etten *et al.*, 1988). It has been known that a 33kDa gene was most abundant *in vitro* translation product and the promoter can work as a strong promoter in eukaryotic expression system (Graves and Meints, 1992). Therefore, the promoter region of the 33kDa peptide was from a *Chlorella* virus isolated in Korea, YK-1 isolate.

The sequence analysis of the cloned 33kDa gene promoter showed 96%

nucleotide sequence homology to that of PBCV-1 (Fig. 5). Despite of the high sequence homology between them, there were two deletions in the promoter region of YK-1 (Fig. 5). Especially, one of deletion contains 3 repeats of the AAAC sequence that was repeated 15 times in PBCV-1. Although no detailed analysis has been performed, this sequence has been suggested to have an important role in transcription (Graves and Meints, 1992). This suggestion has been supported by the finding that the promoters of the PBCV-1 *33kDa* gene contains no obvious eukaryotic promoter elements. Therefore, investigation on the importance of the repeated sequence and the effect of three deletions in YK-1 isolate on the promoter activity are necessary.

The other sequence introduced into the transformation vector was the 3' UTR of the *33kDa* gene for the transcription termination. The formerly developed *Chlorella* transformation vector pCTV do not have any sequence for the transcription of the target gene (Kim *et al.*, 2001). Therefore, two transcripts, one containing the target gene and the Sh ble gene started from CaMV 35S promoter and another transcript for Sh ble gene started from RBC2S promoter are expected to be produced from transformed *Chlorella*. In order to separate the target gene and Sh ble gene, the 3' UTR of the *33kDa* gene of YK-1 isolate was cloned between the EPO gene and RBC2S promoter. Sequence analysis of cloned 3' UTR *33kDa* gene of YK-1 isolate showed 88% homology to that of PBCV-1 (Fig. 10). The TTTTTTTT sequence that has been suggested as a transcription termination sequence of vaccinia virus was found in the PBCV-1 but was not found in YK-1. Instead, the TATATTTT sequence was present at this location. Other sequences of interest found in this region were the TGATTGAA motif that is repeated twice in both PBCV-1 and YK-1 and the ATGTAAAAA motif that is repeated twice in the PBCV-1 but once in YK-1. However, the

importance of these motifs in transcription termination is not known yet.

Stable integration of introduced DNA in chromosomal DNA is prerequisite for stable expression of introduced target gene. The presence of introduced DNA was confirmed in two ways. First, the transformed *Chlorella* was cultured in the presence of selective antibiotic, phleomycin. Cell growth was detected only from *Chlorella* cells transformed with three constructed transformation vectors but not from non-transformed cells. This indicated the presence and expression of introduced Sh ble gene. The integration of introduced DNA was further confirmed by PCR amplification of EPO gene from genomic DNAs isolated from the transformed cells. PCR product with expected size was detected from only the transformants but not from non-transformed cells. The PCR products specifically hybridized to EPO specific probes in Southern blot analysis. All these results suggest stable integration of the introduced DNA into the *Chlorella* chromosomal DNA and functional express of Sh ble gene in the transformed cell.

The activity of the *33kDa* gene promoter and the effect of the 3' UTR on the transcription and target gene expression was tested by ELISA assay. The expression of the EPO gene in the transformed cells was detected by this method but further comparison of the expression level is necessary.

국문초록

배양이 용이하고 간단한 대사경로를 가지는 진핵 미세 조류인 *Chlorella*는 이러한 장점으로 인해 산업적 의학적으로 유용한 물질을 생성키 위한 bioreactor로서의 가능성이 제시 되어왔다. 최근, CaMV 35S promoter 및 선택표지로 phleomycin에 내성을 가지는 유전자를 암호화한 sh ble gene을 이용하여 제작한 vector를 이용하여 어류성장호르몬 유전자를 *Chlorella* 형질전환 vector를 개량하여 *Chlorella*에서 발현시킨 바 있다. 외래 유전자를 대체하여 *Chlorella* 내에서 보다 안정적이며 높은 발현율로 발현시킬 수 있는 체제의 vector로 변형하였다. 우리나라에서 분리한 *Chlorella virus strain* 중 YK-1 isolation에서 33kDa gene promoter 및 전사율 조절에 관여할 것으로 예상되는 3' UTR을 분리하여 새로운 vector를 제작하였다. 분리된 33kDa gene promoter는 기존에 보고된 *Chlorella virus strain*인 PBCV-1의 그것과 98% 염기서열의 상동성을 가졌다. PBCV-1이 가지는 AATAAAT 염기서열이 존재하지 않았지만 promoter function에 관여할 것으로 보이는 AAAC 염기서열은 PBCV-1보다 한 번 적은 15번이 반복되어 있었다. 분리된 YK-1 33kDa 3' UTR은 PBCV-1과 88%의 염기서열 상동성을 가지는 것으로 나타났고, transcription termination에 관여하는 5' -TTTTTNT는 존재하지 않았다. 그러나 PBCV-1에는 존재하지 않는 5'-ATTCAGGGTGATGAAATG sequence 가 존재하는 것으로 나타났다. 분리 promoter, 3' UTR, 그리고 사람의 erithropoietin(EPO) 유전자를 이용하여 3 종류의 완성된 vector를 개발하였다. 제작된 vector 는 각각 33kDa gene promoter, EPO gene을 포함하는 mp335EPO vector, 이 vector에 33kDa 3' UTR을 재조합한 vector인 mp3353EPO, 기존 vector에 있는 CaMV 35S promoter에 EPO gene 및 3' UTR을 가지는 mp35S33pEPO vector를 제작하여 각각 *Chlorella*에 형질전환 시켰다. 형질전환된 *Chlorella*로부터 genomic DNA를 분리하여 PCR을 수행한 결과 EPO gene이 chromosomal DNA에 integration 된 것을 확인할 수 있었다. 형질전환시킨 *Chlorella*에서 추출한 총 단백질을 ELISA로 EPO protein의 발현 여부를 확인한 결과 형질전환시킨

*Chlorella*에서만 EPO protein의 발현을 검출할 수 있었다. 그러나 ELISA 방법으로서는 각 vector 특성에 따른 발현 정도의 차이를 정확히 확인할 수 없었다.

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