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Isolation and Characterization of Chlorella Virus from Fresh Water in Korea and Application in Chlorella Transformation System

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Chlorella viruses are large icosahedral, plaque-forming, dsDNA viruses that infect certain unicellular, chlorella-like green algae. The genomic DNA of over 300 kb contains many useful genes and promoters. Over 40 chlorella viruses have been isolated from fresh water in Korea since 1998. The viruses were amplified initially in chlorella strain NC64A, and pure isolates were obtained by repeated plaque isolation. SDS-PAGE analysis revealed similar but distinct protein patterns, both among the group of purified viruses and in comparison with the prototype chlorella virus PBCV-1. Digestions of the 330- to 350-kb genomic DNAs with 10 restriction enzymes revealed different restriction fragment patterns among the isolates. The tRNA-coding regions of 8 chlorella viruses were cloned and sequenced. These viruses contain 14-16 tRNA genes within a 1.2- to 2-kb region, except for the SS-1 isolate, which has a 1039-bp spacer in a cluster of 11 tRNA genes. Promoter regions of several early genes were isolated and their activities were analyzed in transformed chlorella. Some promoters showed stronger activity than commonly used CaMV 35S promoter and chlorella transformation vectors for heterologous protein are beings constructed using these promoters.

Keywords : chlorella virus, microalgae transformation, Phycodnaviridae, promoters, tRNA

Viruses that infect algae are widely distributed in nature and they have been isolated from freshwater and seawater sources throughout the world. Viruses or virus-like particles have been reported in at least 44 taxa of eukaryotic algae since the 1970s (van Etten et al., 1999). However, most of these viruses are not well characterized because they are difficult to obtain in large quantities.

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Paramecium bursaria chlorella virus-1 (PBCV-1) is the most highly studied virus of those found in algae, because it can be produced in large quantities by using exsymbiotic chlorella-like green algae that are derived from *P. bursaria*. PBCV-1 is the prototype of the *Chlorovirus* genus in the *Phycodnaviridae* family, which encompasses large, polyhedral, plaque-forming algal viruses (van Etten, 1995, 2000). In addition to the chlorella viruses, large DNA viruses that infect marine algae have been reported, including MpV viruses, which infect the unicellular alga, *Micromonas pusilla* (Cottrell and Suttle, 1991; Mayer and Taylor, 1979), and EsV viruses and FsV viruses, which infect the filamentous brown algae of the *Ectocarpus* sp. (Muller et al., 1998) and *Feldmannia* sp. (Henry and Meints, 1992), respectively. Recent studies showing that these viruses are involved in the disappearance of algal blooms suggest that they play important roles in aquatic environments (Castberg et al., 2001; Nagaski et al., 1999), and that they can be used for the control of toxic red algae.

DNA sequence analysis of the 330, 742-bp PBCV-1 genome revealed over 700 open reading frames (ORFs), of which 375 were protein-encoding genes (Kutish et al., 1996; Li et al., 1995, 1997; Lu et al., 1995, 1996). The viral-encoded proteins include transcriptional and translational factors, restriction/modification enzymes, topoisomerase, chitinase, and hyaluronan synthase (Graves et al., 1999; Lavrukhin et al., 2000; Sun et al., 1999; Xia et al., 1986; Yamada et al., 1993; Zhang et al., 1998). Interestingly, PBCV-1 genome contains 11 tRNA genes, which indicates that some components of the host-protein synthesis machinery might be replaced by virus-encoded tRNAs (Li et al., 1997; Nishda et al., 1999b). These tRNA genes are clustered in the genome, and may contribute to the preferential translation of viral proteins during the virus replication cycle. In addition, the chlorella viruses represent a novel source of promoters for expressing genes in foreign hosts. For example, the upstream region of the viral adenine

methyltransferase gene functions extremely well in several higher plants and in many bacteria (Mitra et al., 1994).

Chlorella is a unicellular green alga, which has been widely used in aquaculture and food industry. It can be cultured inexpensively in large scale because it requires only a limited amount of minerals and sunlight. Some species grow relatively fast, dividing 2-9 times per day depending on the light intensity and temperature (Sorokin and Krauss, 1958). Chlorella is a eukaryote and therefore can synthesize complex proteins that require post-translational modification in order to become biologically active. These characteristics provide a rationale for the using chlorella as a new system for foreign protein expression. Despite these advantages, attempts to transform chlorella have been limited. Jarvis and Brown (1991) detected transient expression of firefly luciferase in protoplasts of *C. ellipsoidea*. Stable transformants were recovered from nitrate reductase deficient *C. sorokiniana* mutants transformed with nitrate reductase gene from *C. vulgaris* (Dawson et al., 1997). Recently, Hawkins and Nakamura (1999) expressed human growth hormone in transformed chlorella, but the expression was not stable and the biological activity of the expressed protein was not tested.

We have isolated and characterized chlorella viruses from fresh water in Korea and analyzed their genomes and proteins. In addition, we have developed chlorella transformation system to express useful genes using this attractive organism. In order to optimize the expression system, several promoters from the chlorella viruses isolated in Korea were tested with the chlorella transformation system.

Isolation of chlorella virus in Korea

Freshwater samples were collected rivers, ponds, streams. The samples were filtered with approximately 20-kPa vacuum through a 0.22-μm pore-size, Al_2O_3 Anodisk-25 membrane filter (Whatman, USA) that was backed by a glass-fiber filter. The filtrates were kept at 4°C before inoculation into chlorella host. Chlorella strain NC64A kindly provided by Dr. James L. van Etten, of the Department of Plant Pathology, University of Nebraska, Lincoln, NE, USA were cultured in modified Bold's basal medium (MBBM) as previously described (van Etten et al., 1983a). Initially, we attempted to isolate viruses from these samples using plaque assay methods (van Etten et al., 1983b). Filtered water (100 μl) was mixed with 400 μl of fresh chlorella (4.0×10^8 cells/ml) and 2.5 ml of 0.75% agar, and overlaid on a 1.5% MBBM agar plate. The plates were incubated at 25°C for five days. No plaques were recovered using this experimental method. Therefore, we used an alternative method, in which viruses were isolated after amplification of the viral population in the water sample. For the amplification of viruses, 5 ml of filtered water was added to 100 ml of chlorella strain NC64A, which was grown in MBBM medium, and incubated for five days at 25°C with continuous light and shaking at 150 rpm (van Etten et al., 1983a). After complete lysis of the chlorella culture, the supernatant was diluted to 10^{-6}-10^{-7} and 5 μl was plated as described above. Single plaques were picked and inoculated into 100 ml of a fresh chlorella culture. The steps following lysis of the initial chlorella culture were repeated three times. The isolated virus amplified chlorella strain NC64A and purified as described before (van Etten et al., 1983a).

Twenty-three viruses from 9 cities in Korea formed plaques on chlorella strain NC64A and were single-plaque isolated. Although most of the sampling sites were greenish in appearance, the collected samples were not very turbid. To date, the only known hosts for chlorella viruses are the chlorella strain NC64A and the chlorella strain Pbi (van Etten et al., 1991). Strain NC64A exists as a hereditary endosymbiont in green isolates of the protozoan *P. bursaria* (van Etten et al., 1985a). The detection of NC64A-infecting viruses at different sites indicates the presence of similar or related chlorella strains in freshwater throughout Korea.

Characteristics of chlorella viruses isolated in Korea

The plaque size of the Korean chlorella virus was compared with that of PBCV-1. The plaques ranged in diameter from 2 to 5 mm after 7 days at 25°C in a sealed agar plate. The plaque size of PBCV-1 was about 3 mm and the Korean chlorella viruses could be separated into three groups. The first group produced plaques that were smaller than those made by PBCV-1 (YK, HD-2, KH-2, SS-2, BM-2, BL-2, HOS-2, JH-2), and the second group produced plaques that were the same size as those of PBCV-1 (BM-1, NW-2, SW). The third group produced larger plaques (DJ, HS-1, HS-2, HD-1, KH-1, SS-1, BL-1, HOS-1, JH-1, NW-1, ES-1, ES-2, data not shown). It has been reported that the plaque size of chlorella virus is related to the time required for replication of the virus, such that fast-replicating viruses produce larger plaques (van Etten et al., 1991).

The structure of the isolated DJ virus was observed with an electron microscope (Fig. 1). The virus particles in infected chlorella cells were polyhedral and 150-190 nm in diameter, which are typical features of *Phycodnaviridae* viruses. Virus particles with thin outer shells and electron-dense cores were observed using thin sections and negative staining. The host range and morphological characteristics indicate that the isolated viruses belong to the *Phycodnaviridae* family

The structural proteins of the purified viruses ranged in size from 10 kDa to over 200 kDa, according to SDS-

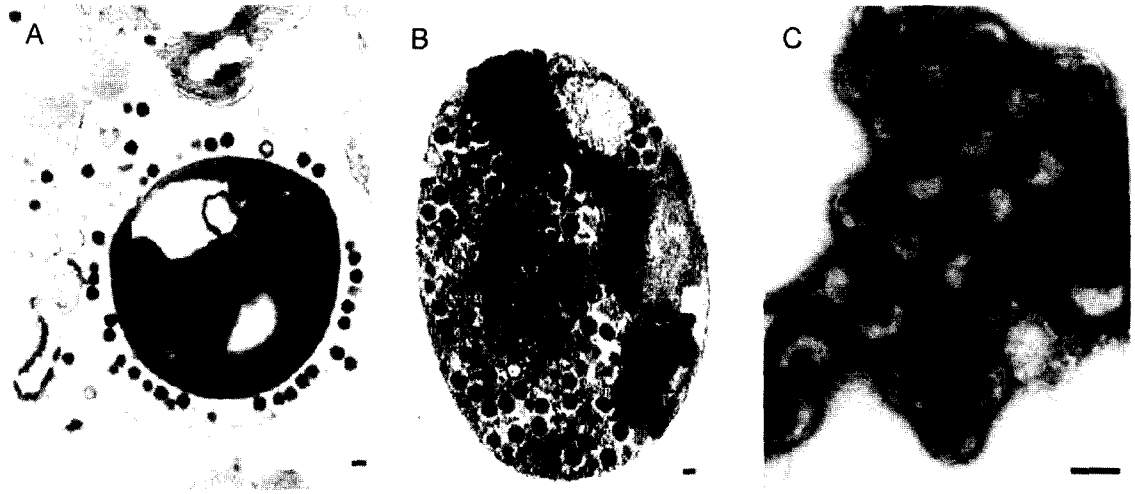

Fig. 1. Electron micrographs of virus-infected cells and negatively stained purified *Chlorella* virus DJ. **A.** Cells at the initial stage of virus infection. **B.** Virus particles have accumulated in the cytoplasm. **C.** Negatively stained purified virus. Scale bars represent 100 nm.

PAGE analysis. The PBCV-1 virus particles contain more than 50 polypeptides, which range in size from 10 kDa to 280 kDa (Skrdla et al., 1984). The 54-kDa major capsid protein, which constitutes about 40% of all the viral proteins, was distinctive, along with a number of minor proteins. The protein sizes and relative content were similar in all of the tested isolates. Nevertheless, the viruses could be divided into several groups based on the comparison of proteins with molecular weights of 25-35 kDa. The PBCV-1, DJ, SS-1 and HS-2 viruses had two proteins within this range, but the HS-2 isolate produced larger proteins than the rest of the isolates. The HS-1, YK, SS-2, KH-1 and KH-2 isolates had three proteins within this range but the KH-2 isolate showed a different band pattern than the other isolates. The *Phycodnaviridae* family encompasses a large number of heterologous viruses. Recent molecular studies have shown that some members of this family lack genes that are present in other chlorella-infecting viruses (Graves et al., 1999; 2001), and that mutant strains exist that have large deletions in their genomes (Landstein et al., 1995). Therefore, differences in the structural protein profiles may or may not reflect molecular diversity, and further characterization of the genomic DNA is necessary for the detailed classification of these viruses.

The genomic DNAs of 8 Korean isolates were digested with 10 different restriction enzymes. In addition to differences in sensitivity to digestion, there were significant differences in the DNA fragment patterns among these isolates (Fig. 2). However, as is the case with the protein profiles, restriction fragment length polymorphism (RFLP) analysis has not been applied to the sub-grouping of these viruses. One of the unusual features of chlorella virus DNA is that it contains relatively high levels of methylated bases (van Etten et al., 1985b). The genomic DNA of the SS-1

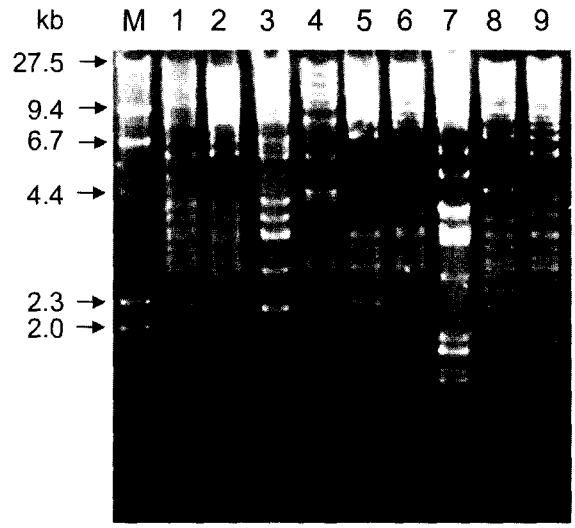

Fig. 2. Restriction fragment length polymorphism analysis of *Chlorella* virus genomic DNAs that were digested with *Bam*HI lane M, DNA size marker; lane 1, PBCV-1; lane 2, DJ; lane 3, HS-1; lane 4, HS-2; lane 5, YK; lane 6, SS-1; lane 7, SS-2; lane 8, KH-1; lane 9, KH-2.

isolate was resistant to digestion with the restriction enzymes *Hin*dIII, *Alu*I and *Pvu*II, These restriction endonucleases all recognize the sequence AGCT, and all are inhibited by the methylation of the first cytosine residue. The SS-1 DNA was also resistant to *Hae*III, which recognizes and cleaves GGCC motifs, but which is also inhibited by the methylation of the cytosine residue. The gene encoding the GpC methylase has been cloned from chlorella virus (Xu et al., 1998). The gene encoding the AGCT methylase has been cloned from *Arthrobacter luteus* (Zang et al., 1993), but not from any chlorella virus. The fact that all of the above enzymes are inhibited by methyl-

ation of the cytosine residue suggests that SS-1 encodes the GC or the AGCT methylase or both. Although the isolated viruses were morphologically similar and shared a common host, the observed protein patterns and sensitivities of the genomic DNAs to restriction enzymes suggest that there are several distinct chlorella viruses in Korea.

Structure of the tRNA gene clusters chlorella viruses

The number and arrangement of the tRNA genes was different in each virus which could be used in the grouping of theses viruses. Therefore, the tRNA-encoding regions of eight chlorella viruses isolated in Korea were PCR amplified with primers that were based on the PBCV-1 sequence. The forward primer (5'-GATAGGGTATGCAA-GTGGTC-3') and reverse primer (5'-GATCCCTGCAGG-TTTCGATC-3') correspond to the 5'-end of the first leucine tRNA gene and the 3'-end of the last valine tRNA gene of the PBCV-1 gene cluster, respectively. The Ile-c primer (5'-TTGGCTCATAAGACCAATGC-3') and the val-n primer (5'-GATCGAAACCTGCAGGGATC-3') were designed to confirm the 5'- and 3'-flanking regions of the tRNA region, respectively. The Gly-N primer (5'-TAACAGCCTTCC-AAGCTGTA-3') and the Arg –N primer (5'-GCGACAGA-CTTCTAATCTGT-3') were used for sequencing of the internal region of the tRNA gene cluster. The primers were located in the first leucine tRNA and the last valine tRNA of PBCV-1. However, there was another leucine tRNA gene in the PBCV-1 tRNA gene cluster at the third position, and two PCR products of 1.2-2 kb were detected in all of the tested viruses, which indicated the presence of more than one leucine or valine tRNA species in this region. Both PCR products were cloned into the pGEM-T vector and their sequences were determined. The sequence analysis revealed the presence in this region of 11 to 16 tRNA genes, which were separated by small spacers (Fig. 3). Genes that encode tRNA have also been identified in the genomes of murine gamma herpesvirus 68 (Bowden et al., 1997) and bacteriophages T4 and T5 (Calendar, 1988; Desai et al., 1986). Nonetheless, the presence of tRNA genes in viral genomes is unusual, considering the general dependence of viral translation processes on the host translational machinery. However, the presence of viral tRNA genes can be explained by the codon usage bias of chlorella viruses and their hosts (van Etten et al., 1991). This bias was predicted from differences in the G+C contents of the two organisms, i.e., 40% G+C for PBCV-1 genome and 67% G+C content for the genome of chlorella NC64A (van Etten et al., 1999). In addition, Nishida et al. (1999b) identified 14 tRNA genes in chlorella virus CVK2, and showed that these tRNAs were expressed and amino-acylated, which indicates that they are involved in viral DNA synthesis.

An interesting feature of the tRNA regions is the presence of a 1039-bp spacer between the arginine (UCU) tRNA and the valine (AAC) tRNA of the SS-1 virus. This spacer comprises an 885-bp ORF that encodes a putative protein of 294 amino acids with a molecular weight of

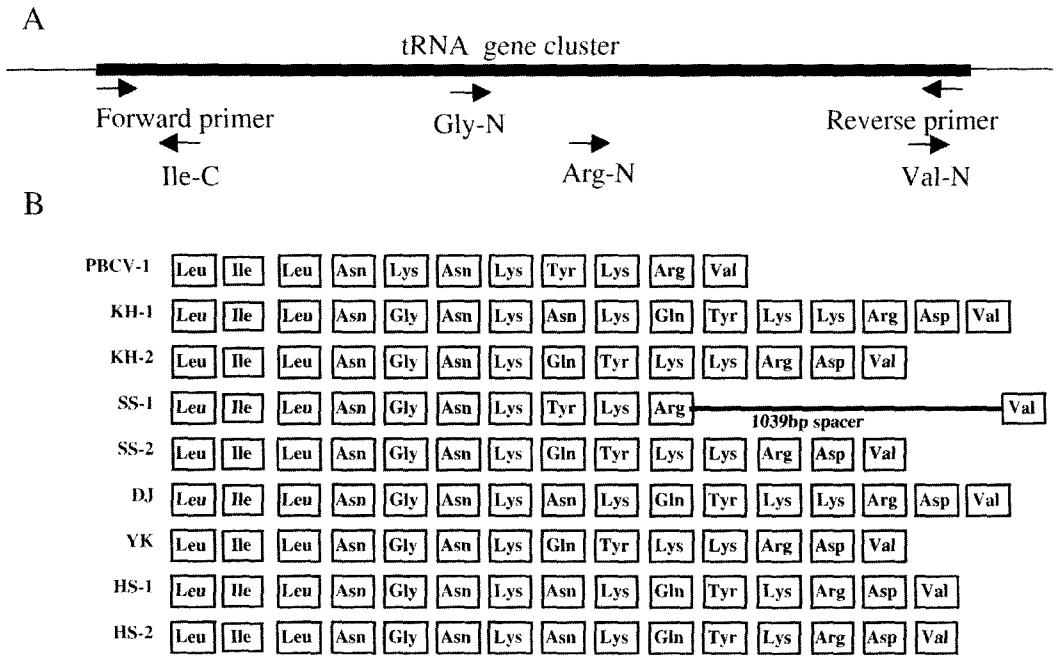

Fig. 3. Arrangement of tRNA genes in different *Chlorella* viruses. **A.** The relative location and orientations of primers used for PCR and sequencing. **B.** The arrangement of the tRNA genes of selected viruses that were isolated in Korea.

about 29 kDa. Sequence comparisons showed 51% amino acid sequence similarity between the spacer ORF and PBCV-1 ORF A478L. It has been suggested that re-arrangements of chlorella virus genomes occur dynamically and frequently during replication in natural environments (Nishida et al., 1999a). For example, a total of 29 of the PBCV-1 ORFs resemble one or more of the other PBCV-1 ORFs, suggesting that they belong to gene families or that they may have arisen by gene duplication (van Etten et al., 1999).

Development of chlorella transformation system

Although chlorella is very attractive as bioreactor for foreign protein expression, there has been no report about stable transformation of this organism. We have developed a transformation vector fro chlorella transformation and successfully expressed flounder growth hormone (fGH), which showed biological activity. The vector was constructed by modifying a plant transformation vector pMinGFP contains the oriV origin for replication in *E. coli* and *Agrobacterium*, the npt II gene for kanamycin resistance, the trfA gene for DNA replication, and the right and left border of *Agrobacterium* derived T-DNA elements, and the green fluorescent protein (GFP) gene under the control of CaMV 35S promoter. After a test of chlorella transformation, the GFP gene in the pMinGFP vector was replaced with the flounder growth hormone gene.

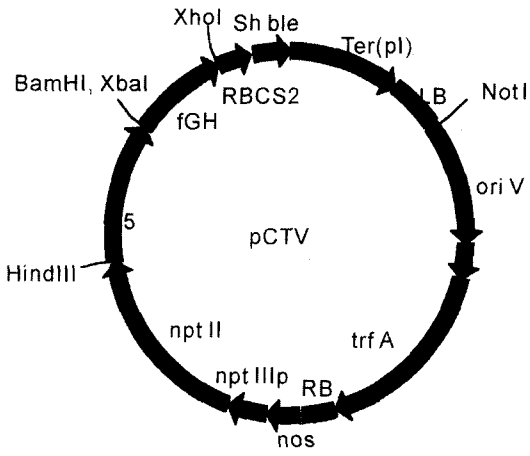

Fig. 4. Schematic diagram of the transformation vector pCTV. In this vector, the fGH gene is under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the *Sh ble* gene is under the control of the *Chlamydomonas* RBCS2 gene promoter. Ori V, npt IIIp-trfA, RB, LB, nos-npt IIIp-npt II, Ter(pI) represent broad host range bacterial replication origin, the chimeric trfA gene with npt III promoter, right border region, left border region, nptII gene under control of nos-npt III double promoter, 3' region of the potato protease inhibitor II gene, respectively. pMinGFP contains the GFP gene instead of fGH, and does not have the RBCS2-*Sh ble* fragment. pMinfGH is the same as pCTV except it lacks the RBCS2-*Sh ble* fragment.

The transformation vector pMinGFP contains the npt II gene for kanamycin resistance but there was no growth inhibition of non-transformed chlorella up to 250 μg/ml Kanamycin. Natural resistance of chlorella species to ampicillin, chloramphenicol, and streptomycin has been reported (Hawkins and Nakamura, 1999). We have tested another antibiotic, phleomycin, and detected complete growth inhibition at the concentration of 1 μg/ml. Therefore, we introduced the *Sh ble* gene under the control of *Chlamydomonas reinhardtii* RBCS2 promoter into the transformation vector to confer resistance to phleomycin, which resulted in the vectór pCTV (Fig. 4; Kim et al., 2002).

The DNA vector was introduced into chlorella protoplast using polyethylene glycol. After transformation and cell wall regeneration, chlorella cells were transferred to f/2 medium containing 1 μg/ml phleomycin. There was detectable growth after 5 days and the growth reached stationary phase by 15 days. The transformed cell showed same growth as non-transformed cell in the presence of 1ß¹/ß¢of phleomycin. The presence of the introduced DNA was determined by PCR and Southern blot analysis using genomic DNA isolated from transformed chlorella. The expression of the introduced fGH gene was tested by immunoblot analysis.

Biological activity of the fGH protein expressed in chlorella was demonstrated by feeding it to flounder fry. Because fish can does not have cellulose that digest the chlorella cell wall, chlorella was first feed to two species of zooplankton, brine shrimp (*Artemia nauplius*) and rotifer (*Brachionus plicatilis*). Immunoblot analysis confirmed that the fGH expressed in chlorella cells accumulated in the zooplankton body until 1 hr feeding, then started to degrade and disappeared 2 hrs after feeding. Four-day-old flounder fries were cultured for 30 days with zooplankton enriched for 1 hr with transformed or non-transformed chlorella. Flounder fries cultured with transformed chlorella for 30 days exhibited a 25% increase in both total length and width. This results shows that foreign protein can be expressed in transformed chlorella cell as biologically active form.

Modification of the chlorella transformation vector using promoters from chlorella viruses

Even though it has been reported that CaMV 35S promoter works in microalgae including *Chlamydomonas* (Dunahay, 1993; Hasnain et al., 1985), the efficiency of this promoter has not been compared with other promoters that function in chlorella. 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 and hyaluronam synthase (Graves et al., 1999; Lavrukhin et al., 2000; Sun et al., 1999; Xia et al., 1986; Zhang et al., 1998). In addition, several putative promoter sequences have been identified in the PBCV-1 genome characterized by high A+T content up to 80% (Schuster et al., 1990). We have isolated several promoters from chlorella viruses isolated in Korea, analyzed the sequences and used to modify chlorella transformation vector pCTV that we had developed.

Two factors have been considered for choosing the promoters; the amount of protein produced in virus infected chlorella cell and the time of gene expression. Although other factors can affect the amount of protein expressed, the expression level is related to the transcription level of the specific gene, which reflects the activity of the promoter. The other consideration is the time of expression. It is known that there is no gene encoding a RNA polymerase in the genome of chlorella viruses, which indicates that the viruses use host RNA polymerase for transcription. However, there are many transcriptional factors encoded by the genome of PBCV-1. One possible explanation for this is that the early genes of this virus are transcribed by host RNA polymerase without modification but the enzyme is modified by viral proteins for the transcription of late genes. Therefore, promoters from early genes can be used for the construction of chlorella transform vector in which the 35S promoter is replaced with these candidate promoters.

One sequence of interest was the promoter for the 33kDa peptide gene (*33kDa*). The biological function of *33kDa* has not been determined, but it is the most abundant protein in the *in vitro* translation of mRNA isolated from PBCV-1 infected cell (Graves and Meints, 1992). This protein was detected at the beginning at 20 min post-infection of PBCV-1 virus. Because this promoter is very strong and since it can be transcribed by host RNA polymerase early in the infection cycle, these were important factors to consider in the search for promoters for the chlorella expression system.

The other factor considered in the vector construction is the termination of transcription. 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. It is possible that, like vaccinia virus, it signals the termination of transcription for PBCV-1 (Schuster et al., 1990).

We modified the pCTV vector by incorporating the promoter and the 3' UTR region of the *33kDa* peptide gene from a chlorella virus. The *33kDa* gene promoter was used to replace the 35S promoter and the 3' UTR was introduced to separate the target gene and downstream *Sh ble* gene and the GFP gene was replaced with human erythropoietin (EPO) gene. Immunoblot analysis and ELISA of proteins from chlorella cells transformed with the modified vector showed same level of protein expression compared to chlorella cells transformed with a vector containing CaMV 35S promoter.

Perspective

Chlorella is an attractive organism for complex recombinant protein production because of its eukaryotic characteristics and low cost for large-scale culture. We have developed a chlorella transformation system and expressed a foreign protein in biologically active form. We also showed that promoters from chlorella virus can be used to express foreign proteins using this system. These days, many human genes of which deficiency can cause serious disease are being identified. By using this cost effective eukaryotic expression system, proteins of medical usage such as EPO that used in this experiment can be produced, and used for treatment of many fetal diseases.

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