韓蠶學誌 40(2), 111~116(1998)
Korean J. Seric. Sci.
<論 文>

Construction of Stably Transformed Bm5 Cells by Using *Autographa californica* Nuclear Polyhedrosis Virus IE1 Gene

Eun Sook Cho[1], Byung Rae Jin[1], Hung Dae Sohn[1], Kwang Ho Choi[1], Soung Ryul Kim[1], Seok Woo Kang[2], Eun Young Yun[2], Sang Hyun Kim[2], Keun Young Kim[2], Yeon Ho Je[3] and Seok Kwon Kang[3]

[1]*College of Natural Resources and Life Science, Dong-A University, Pusan 604-714, Korea.*
[2]*Dept. of Sericulture & Entomology, The National Institute of Agricultural Science & Technology, RDA, Suwon 441-100, Korea.*
[3]*College of Agriculture & Life Sciences, Seoul National University, Suwon 441-744, Korea*

ABSTRACT

To construct transformed Bm5 cells, *Autographa californica* nuclear polyhedrosis virus (AcNPV) IE1 gene, an immediate early viral gene was firstly used in this study. AcNPV IE1 gene, which shares on 95.3% nucleotide sequence homology with *Bombyx mori* nuclear polyhedrosis virus (BmNPV) IE1 gene, was isolated and cloned into pBluescript. Neomycin gene from pKO-neo was inserted under the control of the IE1 promoter to yield pAcIE1-neo. The plasmid pAcIE1-neo was transfected into Bm5 or Sf9 cells, and neomycin-resistant cells were selected in TC100 medium containing 10% fetal bovine serum (FBS) and 1 mg/ml G418 for two weeks. Individual clones were picked and each was amplified for further characterization. The genomic DNA from neomycin-resistant cells was isolated and characterized by PCR using AcNPV IE1 gene-specific primers and by Southern blot analysis using neomycin gene probe. We concluded that AcNPV IE1 gene was functional in *B. mori*-derived Bm5 cells as well as *Spodoptera frugiperda*-derived Sf9 cells to produce stably-transformed insect cells.

Key words : *Autographa californica* nuclear polyhedrosis virus, *Bombyx mori* nuclear polyhedrosis virus, Immediate early gene, Insect cells

INTRODUCTION

Baculoviruses are used widely as vectors for the high-level expression of foreign genes in insect cells (Jin *et al.*, 1995; Luckow and Summers, 1988; Maeda, 1989; O'Reilly *et al.*, 1992). The baculovirus expression vector system majorly utilizes the strong polyhedrin gene promoter of the AcNPV or BmNPV, and the recombinant proteins are produced in insect cells or larvae (Choudary *et al.*, 1995; Luckow and Summers, 1988; Maeda *et al.*, 1985; O'Reilly *et al.*, 1992). The recombinant proteins have been shown, in most cases, to be processed similarly as compared to their authentic counterparts. This feature, together with the high expression levels obtained, have made the baculovirus expression vector system highly attractive. A common insect cell line, *S. frugiperda*-derived Sf9 or Sf21, is used for AcNPV-mediated expression of foreign genes. *B. mori*-derived Bm5 cell line is used for BmNPV-mediated expression of foreign genes. BmNPV expression vector system has also been frequently utilized for high level expression of foreign genes in silkworm larvae. Recently, the host range of AcNPV is expanded by homologous recombination with various methods (Kondo and Maeda, 1991; Mori *et al.*, 1992; Croizier *et al.*, 1994; Jin *et al.*, 1997).

IE1, an immediate early gene of the baculoviruses have been identified (Guarino and Summers, 1987; Huybrechts *et al.*, 1993; Theilmann and Stewart, 1991). IE1 has several known activities, including transactivation of all known early genes (Guarino

and Summers, 1986; Ribiero *et al.*, 1994), down-regulation of expression from other early promoters such as ie0 and ie2 in transient expression assay (Kovacs *et al.*, 1992), and formation of DNA-protein complexes by interacting with the viral enhancer element hr5 (Guarino and Dong, 1991). In contrast, transformed lepidopteran cells were produced by using the AcNPV IE1 promoter, and expressed a foreign gene product continuously in the absence of viral infection (Jarvis *et al.*, 1990). In addition, AcNPV IE1 gene was demonstrated to be transcribe in several lepidopteran cells in transient expression assay (Lu *et al.*, 1996).

In this study we have transfected AcNPV IE1 gene horboring neomycine gene into Sf9 and Bm5 cells. We demonstrated that AcNPV IE1 gene was functional in Bm5 cells as well as Sf9 cells to produce stably-transformed insect cells.

MATERIALS AND METHODS

1. Cell lines and viruses

Sf9 (Vaughn *et al.*, 1977) and Bm5 (Grace, 1962) cells were used in this study. Cells were maintained at 27°C in TC100 medium (Sigma) supplemented with 10% heat-inactivated FBS (Gibco). AcNPV was propagated in Sf9 cells as described previously (O'Reilly *et al.*, 1992; Summers and Smith, 1987).

2. Construction of plasmid by using IE1 promoter

The AcNPV IE1 gene on a 3.1 Kb ClaI-HindIII restriction fragment from AcNPV (Guarino and Summers, 1987) was isolated and cloned into pBluescript SK(-) to yield pAcIE1. The 1.4 Kb HincII fragment in the coding region of IE1 gene was deleted to produce pAcIE1-del. Neomycin gene (1.45 Kb) from pKO-neo was eluted by *HindIII* digestion, filled in with Klenow fragment, and inserted into HincII site of pAcIE1-del to yield expression plasmid pAcIE1-neo.

3. Construction of transformed insect cells

Cell culture dish (35-mm diameter) was seeded with 1×10^6 Sf9 or Bm5 cells and incubated at 27°C for 1 hr to allow the cells to attach. Five microgram of pAcIE1-neo plasmid DNA in 20 mM HEPES buffer and sterile water to make a total volume of 50 μl were mixed in a polystylene tube. Fifty microliter of 100 μg/ml Lipofectin TM (Gibco) was gently mixed the DNA solution, and the mixture was incubated at room temperature for 30 min. The cells were washed twice with 2 ml serum-free TC100 medium. Serum-free TC100 (1.5 ml) was added to each dish. The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubation at 27°C for 5 hr, 1.5 ml TC-100 containing antibiotics and 10% FBS was added to each dish and the incubation at 27°C continued. At 2 days, cells were selected in TC100 medium containing 10% FBS and 1 mg/ml G418 (Gibco) for two weeks. Individual clones of neomycin-resistant cells were picked and amplified for further characterization.

4. Microscopy

Microscopy of neomycin-resistant Bm5 or Sf9 cells in the TC100 medium containing 10% FBS and 1 mg/ml G418 was performed by using inverted phase contrast microscope (Nikon).

5. Genomic DNA extraction from insect cells

The transformed cell clones were cultured in TC100 medium containing 10% FBS and 1 mg/ml G418. Genomic DNA was extracted from the cell pellets (1×10^7 cells) by using WizardTM genomic DNA purification kit according to the manufacturer's instructions (Promega).

6. Polymerase Chain Reaction (PCR)

The IE1 gene specific primers were prepared to 5'TATCGTGTTCGCCATTAGGGCAG3' for the 5' promoter region and 5'GCTGTATTTGTACGTGAGCGTAC3' for the 3' coding region of AcNPV IE1. Genomic DNA was used as template. The reaction was conducted for 250 ng of sample DNA with 2.5 U of Taq DNA polymerase (Promega), 200 nM each deoxynucleotide triphosphate, 100 pM each primer and 3 mM $MgCl_2$ in a final volume of 50 μl. Amplification was accomplished with the DNA Thermal Cycler (Perkin Elmer Cetus) by using the Step-Cycle program set to denature at 94°C for 1 min, anneal at 55°C for 1 min, and extend at 72°C for 1

min, followed by a 4-s-per-cycle extension for a total of 35 cycles. Following amplification, the PCR reaction products were ethanol precipitated, centrifuged at 10,000 ×g for 30 min, and rinsed with 70% ethanol. These DNAs were analyzed by agarose gel electrophoresis.

7. Southern blot

The genomic DNAs were completely digested with restriction enzymes and electrophoresed on a 0.8% agarose gel. The DNAs were transferred to nylon membrane by capillary transfer. Neomycin gene was used as a probe. Probe DNA labeling and detection of immobilized target DNA were carried out according to method recommended by supplier using Southern-lightTM chemiluminescent labeling and detection system (TROPIX, Inc.).

RESULTS AND DISCUSSION

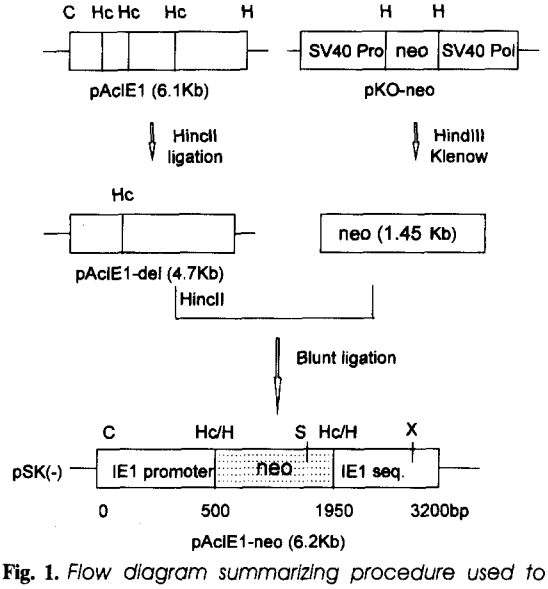

Fig. 1. *Flow diagram summarizing procedure used to generate pAcIE1-neo. AcNPV IE1 gene on a 3.1 Kb ClaI-HindIII fragment was inserted into ClaI and HindIII sites of pBluescript SK(-) to yield pAcIE1 and then 1.4 Kb IE1 coding region of pAcIE1 was deleted using HincII to yield pAcIE1-del. The neomycin gene was excised as HindIII fragment from plasmid pKO-neo, filled in with Klenow fragment and blunt-end ligated to HincII site of pAcIE1-del to generate expression plasmid pAcIE1-neo. C, ClaI; Hc, HincII; H, HindIII; S, SalI; X, XbaI.*

1. Construction of plasmid by using IE1 promoter

The flow diagram summarizing procedure used to generate pAcIE1-neo was described in Fig. 1. AcNPV IE1 gene from AcNPV was cloned into pBluescript. Neomycin gene from pKO-neo was inserted under the control of the IE1 promoter to yield pAcIE1-neo. The plasmid pAcIE1-neo in this study was constructed for the development of the transformed Sf9 cells as reported previously (Jarvis *et al.*, 1990).

2. Construction of transformed insect cells

To construct transformed insect cells, plasmid pAcIE1-neo was transfected into Sf9 or Bm5 cells. The neomycin-resistant cells were selected in TC100 medium containing G418 for two weeks. Significant

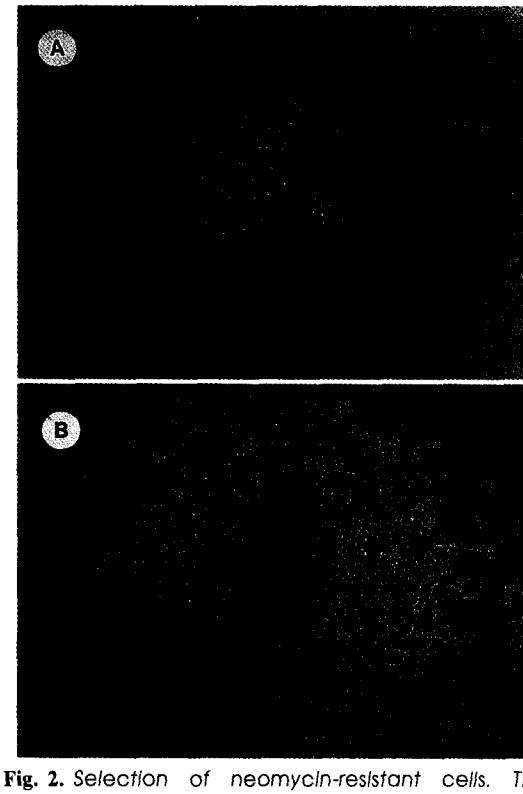

Fig. 2. *Selection of neomycin-resistant cells. The expression plasmid pAcIE1-neo was transfected into Sf 9 and Bm5 cells. After transfection, the cells were selected in TC100 medium containing 10% FBS and 1 mg/ml G418. The neomycin-resistant cell clone from Sf 9 (A) and Bm5 (B) cells was observed by phase-contrast inverted microscope (×200).*

numbers of neomycin-resistant cell clones observed after transfection with pAcIE1-neo, but not in the controls. Individual cell clone was picked in Sf9 (Fig. 2A) and Bm5 (Fig. 2B) cells. In this result, neomycin-resistant Bm5 cell clones were apparently selected in Bm5 cells by pAcIE1-neo. This suggests the neomycin-resistant Bm5 cell clone selected by pAcIE1-neo was due to 95.3% nucleotide sequence homology between IE1 genes of BmNPV and AcNPV (Huybrechts *et al.*, 1992). In addition, although the transformed B. mori-derived cells by AcNPV IE1 gene has not been previously reported, AcNPV IE1 gene was demonstrated to be transcribe in several lepidopteran cells in transient expression assay (Lu *et al.*, 1996). Thus, our result showed that the transformed *B. mori*-derived cells by using AcNPV IE1 gene are constructed.

Fig. 3. *PCR of genomic DNA of the transformed cells. The genomic DNA was extracted from the transformed Sf9 (A) and Bm5 (B) cells. PCR primers were prepared as described in the materials and methods. Lane 1; control cells, Lane 2; transformed cells.*

3. Characterization of the genomic DNA of the transformed cells

To verify presence of neomycin gene in the genomic DNA of the transformed cells, the genomic DNA extracted from the transformed Sf9 and Bm5 cells was characterized by PCR and Southern blot analysis. In the PCR using AcNPV IE1 gene-specific primers, PCR product as expected was observed in the transformed cells, but not in the controls (Fig. 3). The molecular size of the PCR product in the transformed Bm5 cells as well as Sf9 cells was identical to that expected. The PCR

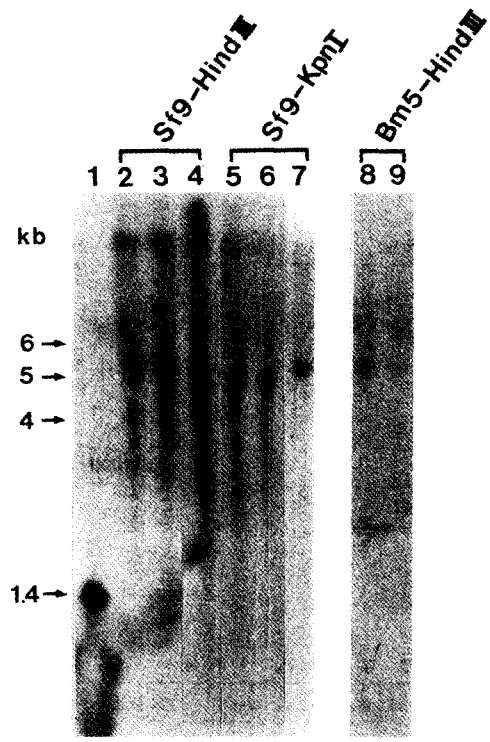

Fig. 4. *Southern blot analysis of genomic DNA of the transformed cells. The genomic DNAs were extracted from the cell clones of the transformed Sf9 (A) or Bm5 (B) cells, and digested with restriction enzymes HindIII and KpnI. The probe was a neomycin gene as described in the materials and methods. Size standards are shown in the reft of panel. Lane 1, probe DNA; Lanes 2 and 5, cell clone #1 from the transformed Sf9 cells; Lanes 3 and 6, cell clone #2 from the transformed Sf9 cells; Lanes 4 and 7, cell clone #3 from the transformed Sf9 cells; Lane 8, cell clone #1 from the transformed Bm5 cells; Lane 9, cell clone #2 from the transformed Bm5 cells.*

product was confirmed by partial nucleotide sequencing and restriction enzyme digestion (data not shown).

In the Southern blot analysis, the genomic DNA of the transformed Bm5 and Sf9 cells was hybridized with neomycin gene probe (Fig. 4). The molecular sizes of the hybridized bands were approximately 5 Kb. This result suggested that AcNPV IE1 gene containing neomycin gene was integrated into chromosome of Sf9 and Bm5 cells (Jarvis *et al.*, 1990).

In conclusion, our results showed that neomycin-resistant cells by pAcIE1-neo were selected from both Sf9 and Bm5 cells, demonstrating that AcNPV IE1 gene was functional in *B. mori*-derived Bm5 cells as well as *S. frugiperda*-derived Sf9 cells to produce stably-transformed insect cells. Thus, this system can be applied for the production of the transformed insect cells of diverse interest.

ACKNOWLEDGEMENT

This work was supported by a grant from the Ministry of Science and Technology of Korea.

적 요

형질전환 누에 세포주를 제작하기 위하여, AcNPV의 초기 발현 유전자 IE1을 이용하였다. 누에 핵다각체병 바이러스 BmNPV IE1과 95.3%의 염기서열 상동성을 가지고 있는 AcNPV IE1 유전자를 클로닝하고, 그 AcNPV IE1 유전자의 프로모터 조절하에 pKO-neo 플라스미드로부터 neomycin 유전자를 도입하여, 이를 플라스미드 pAcIE1-neo라 명명하였다. 그 플라스미드 pAcIE1-neo를 Sf9과 Bm5 세포주에 각각 도입하고, 10% 혈청과 m*l* 당 1 mg의 G418이 함유된 TC100 배지에서 배양하면서 형질전환 세포주를 선발하였다. 그 형질전환 세포주로부터 genomic DNA를 추출하여, AcNPV IE1 유전자 특이 프라이머를 이용한 PCR과 neomycin 유전자를 탐침으로 한 Southern blot 분석으로 neomycin 유전자가 포함된 AcNPV IE 1 유전자가 형질전환 세포주의 chromosome에 삽입되어 있는지를 조사하였다. 결론적으로 본 실험의 결과, AcNPV IE1 유전자는 형질전환 Sf9 세포주 뿐만 아니라 Bm5 세포주 제작을 위해서도 충분히 그 사용이 가능함을 보였다.

REFERENCES

Choudary, P. V., S. G. Kamita and S. Maeda(1995) in Expression of foreign genes in *Bombyx mori* larvae using baculovirus vectors (C. D. Richardson, ed.) pp. 243-264. Humana Press, Totowa, N. J.

Croizier, G., L. Croizier, O. Argaud and D. Poudevigne(1994) Extension of *Autographa californica* nuclear polyhedrosis virus host range by interspecific replacement of a short DNA sequence in the p143 helicase gene. *Proc. Acad. Sci.* **USA 91**: 48-52.

Grace, T. C. D.(1962) Establishment of four strains of cells from insect tissue grown *in vitro*. *Nature (London)* **195**: 788-789.

Guarino, L. A. and W. Dong(1991) Expression of an enhancer-binding protein in insect cells transfected with the *Autographa californica* nuclear polyhedrosis virus IE1 gene. *J. Virol.* **65**: 3676-3680.

Guarino, L. A. and M. D. Summers(1986) Functional mapping of a trans-activating gene required for expression of a baculovirus delayed-early gene. *J. Virol.* **57**: 563-571.

Guarino, L. A. and M. D. Summers(1987) Nucleotide sequence and temporal expression of a baculovirus regulatory gene. *J. Virol.* **61**: 2091-2099.

Huybrechts, R., L. A. Guarino, M. V. Brussel and V. Vulsteke(1992) Nucleotide sequence of a transactivating *Bombyx mori* nuclear polyhedrosis virus immediate early gene. *Biochem. Biophys. Acta* **1129**: 328-330.

Jarvis, D. L., J. G. W. Fleming, G. R. Kovacs, M. D. Summers and L. A. Guarino(1990) Use of early baculovirus promoters for continuous expression and efficient processing of foreign gene products in stably transformed lepidopteran cells. *Bio/Technology* **8**: 950-955.

Jin, B. R., C. J. Ryu, S. K. Kang, M. H. Han and H. J. Hong(1995) Characterization of a murine-human chimeric antibody with specificity for the preS2 surface antigen of hepatitis B virus expressed in baculovirus-infected insect cells. *Virus Res.* **38**: 269-277.

Jin, B. R., H. J. Yoon, P. V. Choudary and S. K. Kang(1997) Characterization of recombinant Autographa californica nuclear polyhedrosis virus (NPV) expressing β-galactosidase gene in both Sf21 and Bm5 cells by *Bombyx mori* NPV p143 helicase gene. *Mol. Cells* **7**: 762-768.

Kondo, A. and S. Maeda(1991) Host range expansion by recombination of the baculoviruses *Bombyx mori* nuclear polyhedrosis virus and *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* **65**: 3625-3632.

Kovacs, G. R., J. Choi, L. A. Guarino and M. D. Summers(1992) Functional dissection of the *Autographa californica* nuclear polyhedrosis virus immediate-early 1 transcriptional regulatory protein. *J. Virol.* **66**: 7429-7437.

Lu, M., R. R. Johnson and K. Iatrou(1996) Trans-activation of a cell housekeeping gene promoter by the IE1 gene product of baculoviruses. *Virology* **218**: 103-113.

Luckow, V. A. and M. D. Summers(1988) Trends in the development of baculovirus expression vectors. *Bio/Technology* **6**: 47-55.

Maeda, S.(1989) in Gene transfer vectors of a baculovirus, *Bombyx mori*, and their use for expression of foreign genes in insect cells (Vol 1, J. Mitsuhashi, ed.) pp. 167-181, CRC Press, Inc., Boca Raton, Fla.

Maeda, S., T. Kawai, M. Obinata, H. Fugiwara, T. Horiuchi, Y. Saeki, Y. Sato and M. Furusawa(1985) Production of human alpha-interferon in silkworm using a baculovirus vector. *Nature* **315**: 592-594.

Mori, H., H. Nakazawa, N. Shirai, N. Shibata, M. Sumida and F. Matsubara(1992) Foreign gene expression by a baculovirus vector with an expanded host range. *J. Gen. Virol.* **73**: 1877-1880.

O'Reilly, D. R., L. K. Miller and V. A. Luckow(1992) Baculovirus expression vectors: a laboratory manual. W. H. Freeman & Co., New York.

Ribeiro, B. M., K. Hutchinson and L. K. Miller(1994) A mutant baculovirus with a temperature-sensitive IE1 transregulatory protein. *J. Virol.* **68**: 1075-1084.

Summers, M. D. and G. E. Smith(1987) in Manual of methods for baculovirus vectors and insect cell culture procedures, Texas Experiment Station Bulletin No. 1555, Texas A & M University.

Theilmann, D. A. and S. Stewart(1991) Identification and characterization of the IE-1 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. *Virology* **180**: 492-508.

Vaughn, J. L., R. H. Goodwin, G. J. Thompkins and P. McCawley(1977) The establishment of two insect cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *In Vitro* **13**: 213-217.