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Construction of a Baculovirus Expression System Using *Hyphantria cunea* Nuclear Polyhedrosis Virus for Eukaryotic Cells

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Abstract Baculovirus transfer and expression vectors with *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) were constructed. An initial transfer vector, pHcEV, constructed using HcNPV was previously reported (Park *et al.* 1993. *J. Kor. Soc. Virol.* **23:** 141-151). Herein, the size of the vector was properly reduced, and a functionally perfect vector was constructed and named pHcEV-IV (6.7 kb). The vector has a 2.2-kb HcNPV DNA sequence in the 5'-flanking region of the vector's polyhedrin gene promoter. The 1.8-kb HcNPV DNA sequence, poly A signal sequence, T3 primer sequence, and 13 multicloning site sequences, in order, were ligated in front of the translation start codon of the polyhedrin gene. The cloning indicating marker *lacZ* gene was inserted into the pHcEV-IV, named pHcEV-IV-*lacZ*, and transferred into the wild-type virus. Recombinant expression virus, *lacZ*-HcNPV, was constructed by replacing the *lacZ* gene in the pHcEV-IV-*lacZ* with the polyhedrin gene of the wild-type virus. The recombinant virus was isolated from blue plaques that produce β-galactosidase without polyhedra. The *lacZ* gene insertion was confirmed by Southern hybridization analysis. The expression of the *lacZ* gene in *Spodoptera frugiperda* cells infected with the *lacZ*-HcNPV was examined by SDS-PAGE and colorimetric assay. One 116-kDa LacZ protein band appeared on the PAGE. The production rate of the β-galactosidase was approximately 50 international units (IU) per min per ml between 2 to 5 days postinfection (p.i.). The highest activity occurred at five days p.i. was 170 IU/min/ml. The enzyme activity first appeared about 20 h p.i. as measured by colorimetric assay.

Key words: Baculovirus, *Hyphantria cunea* nuclear polyhedrosis virus, cloning and expression vector, recombinant virus, *Spodoptera frugiperda* cell, *lacZ*

Nuclear polyhedrosis viruses (NPV) possess a double-stranded, circular DNA genome, with a molecular weight of approximately 8.7×10^7 [2, 13, 22]. They are attractive as vectors for propagating and expressing foreign genes in eukaryotic cells [4, 8, 9, 14, 23, 24, 25, 33, 39] because they have extendable rod-shaped nucleocapsids [5, 17, 32], circular DNA genomes [2], detectable nonessential polyhedrin genes [6, 7, 12, 34], and strong polyhedrin [21, 24, 27] and p10 gene [26, 28] promoters.

Autographa californica MNPV, *Bombyx mori* NPV [23, 33], and *Lymantria dispar* NPV [39] were developed as vectors for eukaryotic cells. The previously reported *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) vector, pHcEV [24], is a 9.5-kb primary vector with only one *Nco*I cloning site. It can be improved by adding multicloning sites and shortening its size. Also, the baculovirus HcNPV expression system was constructed for cloning and expression of foreign genes in the eukaryotic cell system. If the baculovirus expression vector contains a cloning indicator marker for screening, the marker is used in a one-step procedure to transfer the recombinant viruses. Therefore, we undertook this work of constructing an improved baculovirus-eukaryotic expression system.

This article describes the construction of baculovirus transfer and expression vector systems using *Hyphantria cunea* nuclear polyhedrosis virus for expression of foreign genes in insect *Spodoptera frugiperda* cells.

MATERIALS AND METHODS

Virus, Cell Line, and Medium

The plaque-purified clone HL-2 of *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV HL-2) [11] was propagated in *Spodoptera frugiperda* cell line (IPLB-SP-21) [35], with TC-100 medium, as described previously by Lee [11], and Lee and Lee [16].

Bacteria and Plasmids

Escherichia coli JM83 bearing pUC18 clone [36] was used for cloning. *E. coli* XL1-blue/pBluescript SK(+) was used to obtain a multicloning site [30], pCH110 vector was

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used for the *lacZ* gene source [27], and *E. coli* XL1-blue bearing pHcEV plasmid [24] were used for construction of new vectors.

Purification of Plasmid and Viral DNA

E. coli containing recombinant plasmids were cultured in LB broth (1.0% NaCl, 0.5% yeast extract, and 1.0% bacto-tryptone) at 37°C, and then the plasmid DNA was purified by the Birnboim and Doly [1] procedure. Viral multiplication and purification were carried out with the procedure described by Lee and Lee [16].

Restriction Enzyme Digestions and Agarose Gel Electrophoresis

HcNPV genomic DNA and vector DNAs were digested and electrophoresed on 1.0% agarose gel, and the molecular

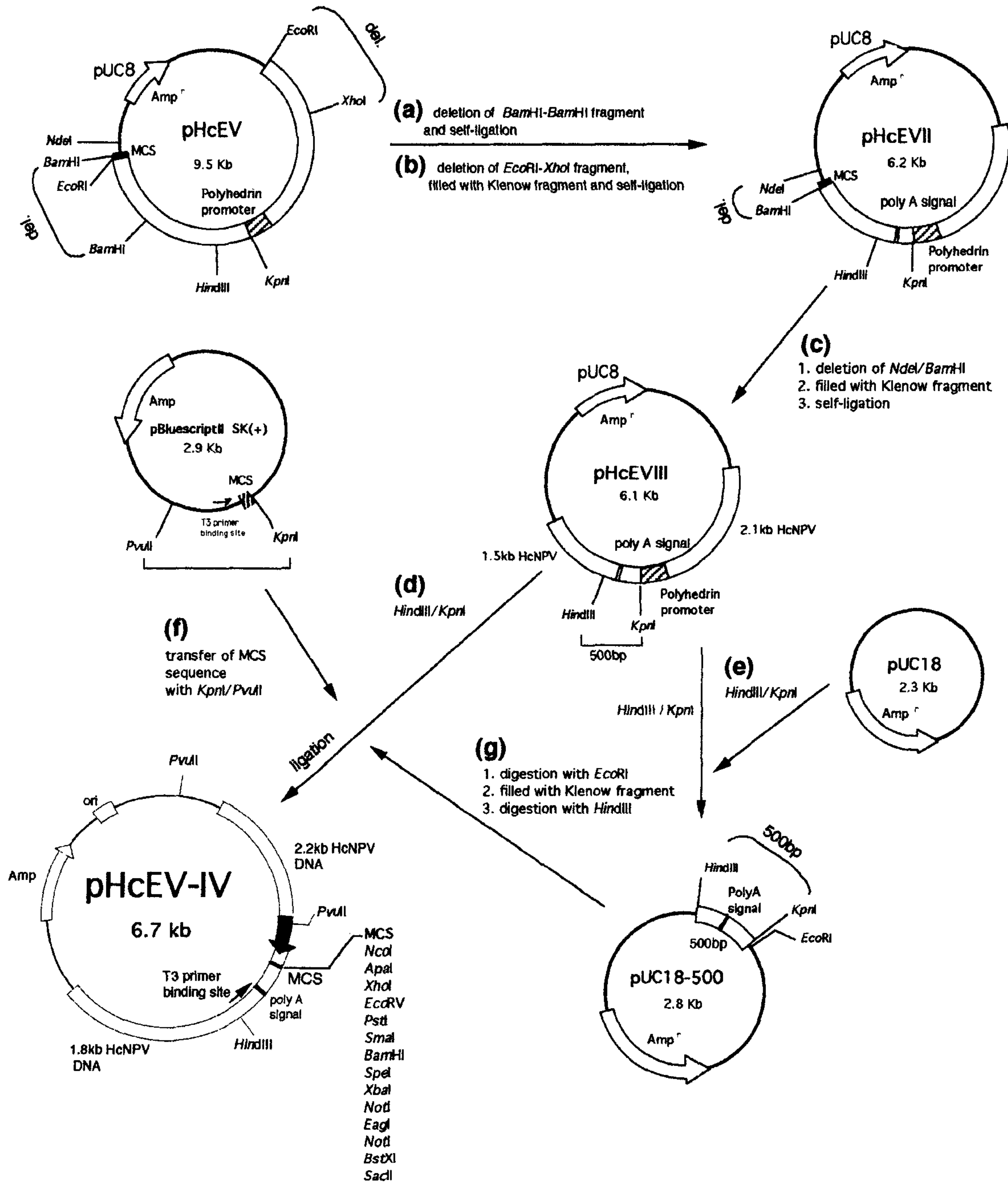

Fig. 1. A scheme of the construction of pHcEV-IV vector.

The pHcEV vector was reduced in its size and the multicloning sites were inserted in front of the translation initiation codon of the polyhedrin gene to construct pHcEV-IV.

sizes of DNA fragments were determined by comparing their mobility with *Hin*dIII-digested phage λ DNA fragments.

Cloning and Transformation

Cloning was carried out by mixing together 15 μl (0.2 μg) of insert DNA, 20 μl (0.1 μg) of vector DNA, 5 μl of 5 mM ATP, 5 μl of 10× T4 DNA ligase buffer, 2 μl (1.8 units/μl) of T4 DNA ligase, and 3 μl of distilled water, and then the total 50 μl mixture was reacted at 14°C for 18 h. The reaction condition was examined by 1.0% agarose gel electrophoresis [13, 14, 19]. DNA from low melting agarose gel was eluted by a slight modification of the procedure described by Weislander [37]. The *E. coli* competent cells were prepared and transformed by the Mandel and Higa method [18].

Construction of Transfer Vectors

A schematic diagram of the reduction of the size of the pHcEV vector is shown in Fig. 1. The primary pHcEV transfer vector (9.5 kb) was progressively modified by the scheme to construct transfer vectors.

Inserting a foreign gene requires the presence of multicloning sites (MCS) [30, 36]. A sequence containing thirteen multicloning sites (380 bp) and the T3 primer region in the vector pBluescript SK(+) [30] were cleaved out, cloned into the pHcEVIII vector, and then transformed into *E. coli* XL1-blue to construct pHcEV-IV vector (Fig. 1). The insertion of the MCS DNA fragment was confirmed by sequencing with the dideoxynucleotide chain termination method of Sanger *et al.* [29].

A cloning indicator marker, the *E. coli lacZ* gene, was inserted into the pHcEV-IV transfer vector. The β-galactosidase (*lacZ*) gene sequence (3.5 kb) of the pCH110 plasmid [27] was transferred into the pHcEV-IV vector.

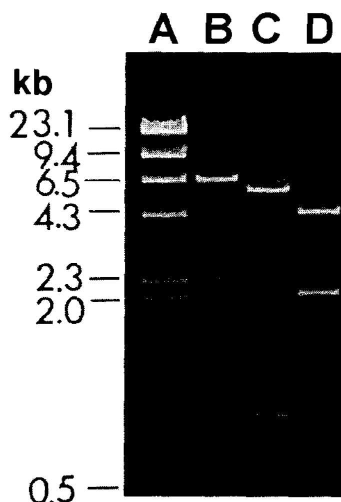

Fig. 2. Restriction pattern of pHcEV-IV vector DNA.

Lanes A, λ DNA digested with *Hin*dIII; B, C, and D, pHcEV-IV DNA digested with *Bam*HI, *Hin*dIII, and *Pvu*II, respectively.

Consequently, a final vector, pHcEV-IV-*lacZ* (10 kb), was constructed by the scheme shown in Fig. 4 and analyzed with restriction enzymes.

Construction of Recombinant Expression Vector Virus

The *S. frugiperda* cells were cotransfected with the transfer vector pHcEV-IV-*lacZ* and the wild-type HcNPV DNA to construct recombinant viruses using lipofectin-mediated transfection, as described by Felgner [3] and Lee *et al.* [14] with modifications. An outline of the construction scheme is shown in Fig. 6. The plasmid DNAs and the viral DNAs were prepared by ultracentrifugation using 25% sucrose cushion (5 mM NaCl and 10 mM EDTA) and phenol extraction. Then, using TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0), the plasmid DNA was diluted to 1.0 μg/μl, and the viral DNA was diluted to 0.5 μg/μl.

Exponentially growing *S. frugiperda* cells (2×10^6 cells) were seeded on a 60×15 mm tissue culture petri

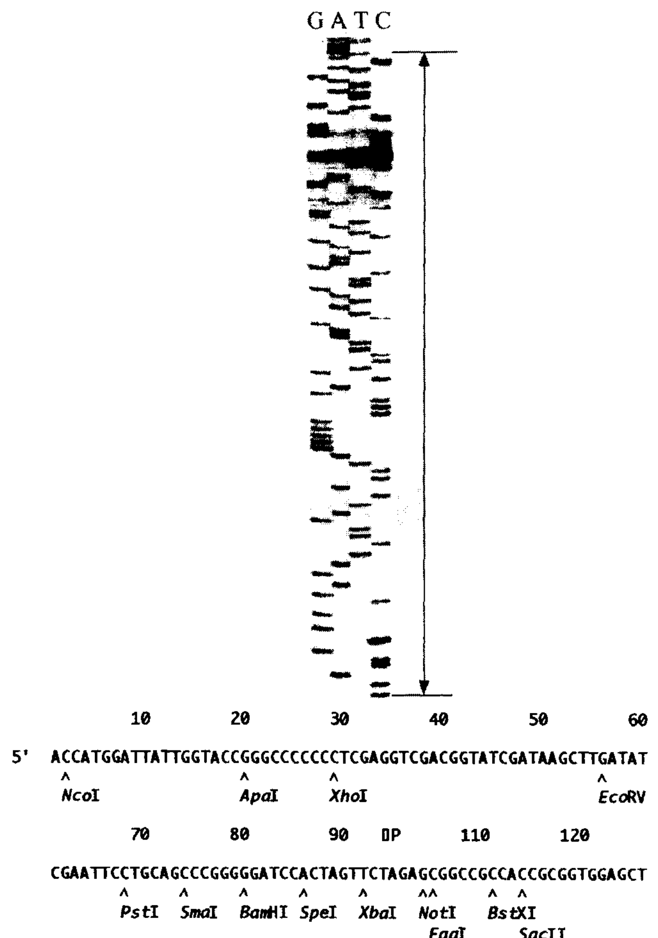

Fig. 3. Map of multicloning sites in the transfer vector pHcEV-IV.

Sequences of the thirteen cloning sites were confirmed with the procedure cleseribed by Sanger *et al.* [29]. The enzyme sites in this multicloming sites are unique in the vector.

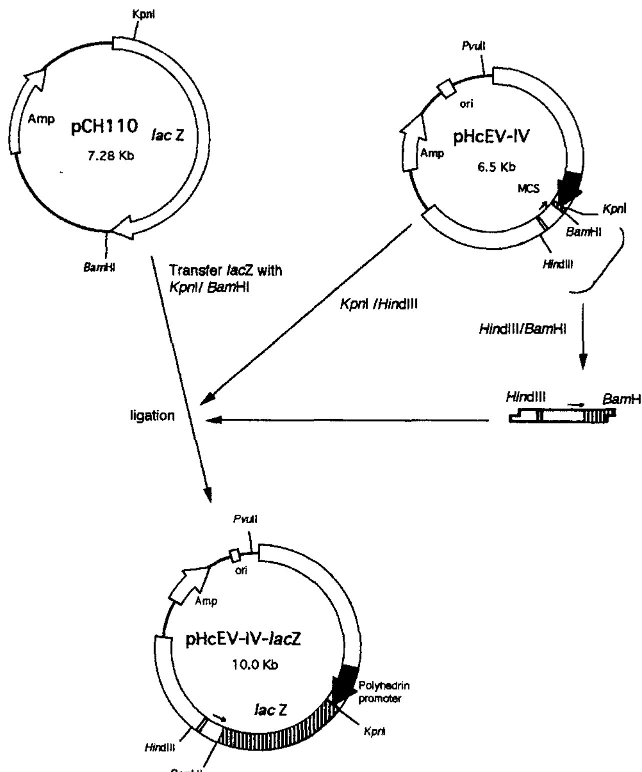

Fig. 4. A scheme of construction of the pHcEV-IV-*lacZ* vector. *E. coli lacZ* gene was transferred into the pHcEV-IV vector.

dish and incubated overnight at 28°C. The old medium was exchanged with 2.0 ml of TC-100 basal medium (without serum and antibiotics). After swirling gently, the medium was removed again, and a 2.5 ml of TC-100 basal medium (without serum and antibiotics) was added and incubated at room temperature for 30 min.

The lipofectin-DNA complexes were prepared in a sterile polystyrene tube. Lipofectin reagent solution was prepared by adding 11 μl of lipofectin (1.0 mg/ml) to 99 μl sterile H_2O in a polystyrene tube. Five microliters of the plasmid DNAs and 5 μl of the viral DNAs in TE buffer were mixed with 40 μl of sterile water in an eppendorf tube. Then, 50 μl of the lipofectin solution was gently mixed in the DNAs tube and incubated at room temperature for 15 min to allow lipofectin-DNA complexes to form.

Meanwhile, the media of the cell monolayers were removed and 2.5 ml of TC-100 basal medium was added. The lipofectin-DNA complexes were added to the cell culture and the dishes were gently swirled to mix the solution. The treated culture was incubated at 28°C for 5 h. Then, 2.5 ml of TC-100 medium containing 10% FBS and antibiotics were added to the dish and incubated at 28°C for 60~72 h. The medium containing recombinant viruses produced in the transfected cells was transferred to a sterile container and stored at 4°C. Recombinant virus was selected by X-gal plaque assay.

Selection of Recombinant Viruses by Plaque Assay with X-gal

Plaque assay was performed to select recombinant viruses containing the *lacZ* gene in their genomic DNAs using a modification of the procedure described by Lee and Miller [17] and Lee *et al.* [14]. The cotransfection supernatant was used as original inoculum for plaque assay and inoculated into *S. frugiperda* cell monolayers in petri dishes (60×15 mm). The method utilizes 1.5% low-melting point agarose (Sea Kem) and a final concentration of 240 μg/ml X-gal to screen the β-galactosidase gene in supplemented TC-100 medium as an overlay. Recombinant virus plaque, which produces blue plaque, was picked from each plate. There was a total of six passages of the virus through the *S. frugiperda* cell.

Confirmation of the *lacZ* Gene on the Recombinant Virus

The *lacZ* gene in the recombinant virus DNA was confirmed by Southern blot hybridization [19, 31]. *Bgl*II restriction DNA fragments of *lacZ*-HcNPV were transferred onto nitrocellulose filter and hybridized with the probe *lacZ* gene DNA. The purified *lacZ*-HcNPV DNA was digested with 15 units of *Bsu*36I enzyme (NEB) for 6 h at 37°C. The *Bsu*36I-digested *lacZ*-HcNPV DNA was digested with *Bgl*II to detect the location of the *lacZ* gene on the *lacZ*-HcNPV. Digestion with *Bgl*II was carried out in the TE buffer (20 mM Tris-HCl, 200 mM NaCl, 1.0 mM EDTA, 10 mM 2-mercaptoethanol, and 50% glycerol) at 37°C overnight, and then run on 0.5% agarose gel at 5 volts for 16 h. The hybridized DNA with the probe DNA was exposed to Hyperfilm™-ECL, and then incubated at room temperature for 10 min. The film was developed manually using Kodak Co. procedure.

The pHcEV-IV-*lacZ* was digested by *Bam*HI and *Kpn*I enzymes, electroeluted, and used as a probe DNA for detection of the *lacZ*-HcNPV recombinant. The probe DNA was labeled with horseradish peroxidase (HRP) using the ECL direct nucleic acid labelling procedure of the Amersharm Co.

Detection of the LacZ Protein by SDS-polyacrylamide Gel Electrophoresis (PAGE) Analysis

The LacZ protein expressed by the *lacZ*-HcNPV was detected with vertical slab SDS-PAGE as described by Laemmli [10]. The cells infected with the recombinant virus were harvested at 48 h p.i., and washed twice with PBS. Cell pellets were resuspended in electrophoresis sample buffer, heated at 100°C for 5 min, and then analyzed by 10% SDS-PAGE. After electrophoresis, proteins were stained with Coomassie Brilliant Blue. Molecular weight standard proteins, myosin (200 kDa), phosphorylase B (97.4 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), and trypsin inhibitor (21 kDa) (Bio-Rad), were used in the analysis.

Assay of β-Galactosidase

Approximately 2×10^6 cells growing exponentially in suspension culture were seeded in tissue culture dishes (100×15 mm) and incubated for 24 h at 28°C for attachment and growth. Then, the cells were infected with the NOVs of the *lacZ*-HcNPV clone at a multiplicity of infection (m.o.i.) of 2. After 1 h of adsorption at room temperature, the monolayers were washed with TC-100 medium. Then, 5 ml of the medium was added to the cells and the culture was incubated for 5 days at 28°C. The cell cultures were collected at 24, 48, 72, 96, and 120 h p.i. The samples were pelleted at 5,000×g for 10 min and then dissolved in 0.25 M Tris-HCl (pH 8.0). Approximately 2×10^6 cells were transferred in 100 μl of 0.25 M Tris-HCl and ultrasonicated three times for 15 sec at 100 μA. The lysates were pelleted at 10,000×g for 5 min, and the supernatants were stored for enzyme assay [20]. Thirty microliters of the supernatant were transferred into 1.0 ml of Z-buffer (0.1 M sodium phosphate buffer pH 7.5, 1.0 mM $MgCl_2$, 45 mM β-mercaptoethanol) and placed at 28°C for 5 min. ONPG (*o*-nitrophenyl-β-D-galactopyranoside, 4 mg/ml) (Sigma Co., St. Louis, U.S.A.) was added to the mixture and incubated at 28°C for 20 min. Then, the optical density was determined with a spectrophotometer (UV-240, Shimadzu) at 420 nm. One unit of β-galactosidase was defined as the amount of enzyme which produces 1.0 nmol of *o*-nitrophenol per min per ml at 28°C, pH 7.0.

RESULTS AND DISCUSSION

Construction of Baculovirus Transfer Vector pHcEV-IV

A previously constructed primary baculovirus *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) transfer vector, pHcEV [24] (Fig. 1), although large, is inefficient to use because it has unnecessary DNA parts, and only one *Nco*I site for cloning of foreign genes. Therefore, its size was reduced by several restriction enzymes according to the construction schematic diagram shown in Fig. 1. In the pHcEV vector, the *Bam*HI-*Bam*HI site sequence (Fig. 1a), the *Eco*RI-*Xho*I site sequence (Fig. 1b), and the *Nde*I and *Bam*HI site sequence (Fig. 1c) are not essential parts of the vector. Therefore, the sequences were removed by enzyme cleavage and the other large sequences were self-ligated. The resulting clones were named pHcEVI with a size of 8.3 kb (Fig. 1a), pHcEVII clone with 6.2 kb (Fig. 1b), and pHcEVIII with 6.1 kb (Fig. 1c). The pHcEVIII clone has 2.2-kb HcNPV DNA upstream and 1.8-kb HcNPV DNA downstream of the HcNPV polyhedrin promoter [24].

The pHcEV-IV vector was constructed by the ligation of the MCS sequence, the T3 primer sequences [30, 36] and the poly A signal sequence into the *Hin*dIII and *Kpn*I sites near the initiation codon of the pHcEVIII clone (Fig. 1d). The vector DNA was analyzed by restriction enzymes, *Bam*HI, *Hin*dIII, and *Pvu*II (Fig. 2). When the vector was cut by the *Bam*HI enzyme, only a single band appeared. This means that the vector has a single *Bam*HI site. Its size is 6.5 kb. *Hin*dIII produced two fragments, 6.0 kb and 0.5 kb, and *Pvu*II produced two fragments, 4.5 kb and 2.0 kb. These results indicated that the size of the whole vector is 6.5 kb.

The orientation and insertion of the MCS sequence in the pHcEV-IV, the MCS area, was confirmed by DNA sequence analysis (Fig. 3), which indicated that the MCS was correctly oriented and inserted in front of the translation initiation codon. Also, the MCS was verified by digestions with thirteen restriction enzymes; *Nco*I, *Apa*I, *Xho*I, *Eco*RV, *Pst*I, *Sma*I, *Bam*HI, *Spe*I, *Xba*I, *Not*I, *Eag*I, *Bst*XI, and *Sac*II. These enzyme sites in the vector have only single cut-site. This result agrees with that of Short *et al.* [30].

Construction of Recombinant Baculovirus *lacZ*-HcNPV

A recombinant *lacZ*-HcNPV using the wild-type HcNPV as a viral vector for expressing foreign DNA in insect cells was developed. When a foreign gene is inserted into the baculovirus, the confirmation and screening of the recombinant may be easy if there is an indicator gene [23]. That is why *E. coli* β-galactosidase (*lacZ*) gene was chosen as the cloning indicating genetic marker. *E. coli lacZ* gene is a very useful marker because expression of *lacZ* gene results in the formation of blue plaque in the presence of an appropriate chromogenic indicator, X-gal [34, 38, 39]. The β-galactosidase (*lacZ*) gene (3.5 kb) was the cloning indicator gene in this study. The *lacZ* gene in the pCH110 vector [27] was digested out with *Kpn*I and *Bam*HI restriction enzymes and then inserted into the pHcEV-IV vector, as reported in the Materials and Methods. The vector was digested with *Nco*I restriction enzyme, treated with Klenow fragment to make a blunt end and then self-ligated, therefore the translation start codon ATG downstream of the pHcEV-IV coincided with the ORF of the *lacZ* gene. This newly generated recombinant plasmid was named pHcEV-IV-*lacZ* (Fig. 4). The pHcEV-IV-*lacZ* DNA was digested with *Bam*HI and *Kpn*I enzymes to confirm the insertion of the gene DNA sequence (Fig. 5). The vector DNA had one *Bam*HI site and was cleaved into 6.5-kb and 3.5-kb fragments by *Kpn*I. The 3.5 kb is the size of the *lacZ* gene DNA fragment, which indicated that the *lacZ* gene was inserted is a proper orientation into the vector.

The pHcEV-IV-*lacZ* transfer vector DNA, and the wild-type HcNPV genomic DNA which has the polyhedrin gene, were cotransfected into *S. frugiperda* cells as described in Materials and Methods (Fig. 6). After cotransfection into the insect cells of the viral DNA with the vector

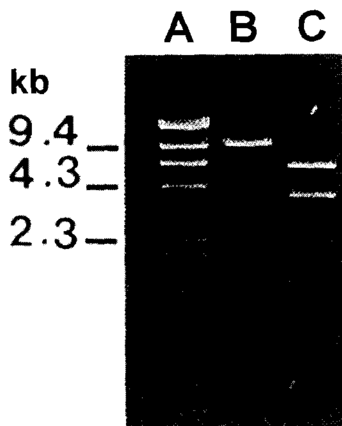

Fig. 5. Confirmation of the insertion of the *lacZ* gene in the pHcEV-IV-*lacZ* vector.

Lanes A, λ DNA digested with *Hin*dIII; B and C, pHcEV-IV-*lacZ* plasmid DNAs digested with *Bam*HI, and *Kpn*I, respectively.

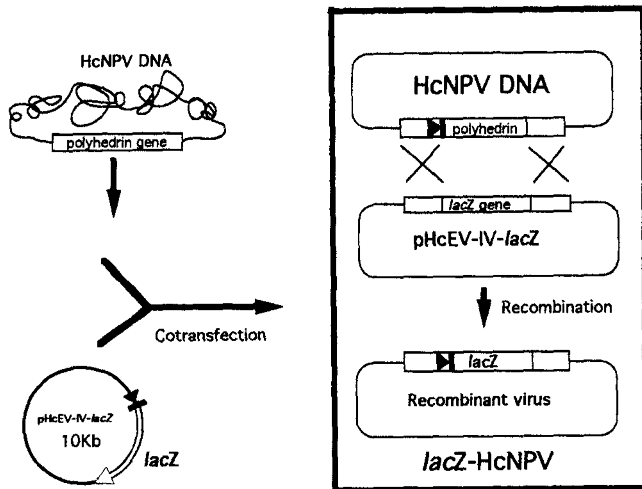

Fig. 6. A recombination strategy to construct a fusion containing polyhedrin promoter and the *lacZ* structural gene by allelic replacement between homologous flanking regions of the HcNPV DNA.

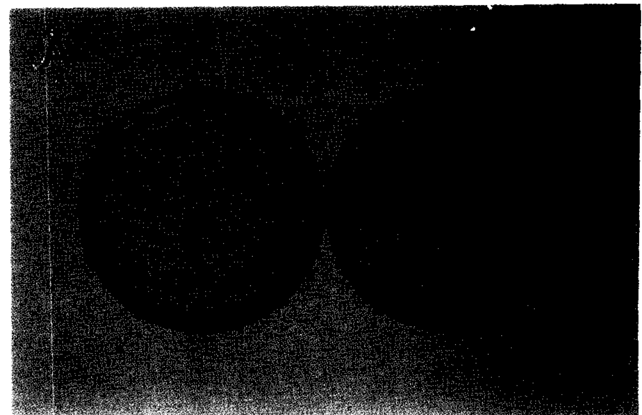

Fig. 7. Plaques on the *S. frugiperda* cell monolayers infected with the *lacZ*-HcNPV overlaid with X-gal-medium.

The diluted *lacZ*-HcNPV was infected on the cell monolayers at 28°C for 1 h and overlaid with 3 ml of TC-100 medium containing 0.2% X-gal and 0.8% low melting point agarose. Panel A: blue plaques formed by recombinant virus *lacZ*-HcNPV at 72 h p.i. The arrow indicates blue plaques. Panel B: magnified plaque (×200). No polyhedra formed in the cells of the plaque.

DNA containing *lacZ* gene, the *lacZ* gene was replaced with the polyhedrin gene in the HcNPV genomic DNA. In this study, the transfer vector and the wild-type viral DNA were successfully cotransfected into *S. frugiperda* cells and multiplied. After 5 days p.i., recombinant viruses in the culture media were plaque-assayed (Fig. 7A). Then, five blue plaque clones on the X-gal plate were isolated and named *lacZ*-HcNPV. Individual clones were named through digital designation on the basis of isolation order. Wild-type virus produces plaques 3 mm in diameter. The recombinant viruses formed plaques 2 to 4 mm in diameter at 28°C. Cells in these plaques contained no polyhedral inclusion bodies (Fig. 7B) which indicated that the recombinant virus did not have a polyhedrin gene. These plaques were plaque-purified twice, and then the largest plaque clone *lacZ*-HcNPV-4 was used. The titer of recombinant virus in the first infected cotransfection supernatant was about 2.0×10^5 pfu (plaque forming unit) per ml at 7 days postinfection (p.i.), and the medium was used for the next infection inoculum. When the recombinant virus was infected to 2×10^6 cells with m.o.i. of 1 at 5 days p.i., the titer was 2×10^8 pfu per ml, the nuclei of the cells were swollen, and the nuclear membranes were hypertrophied to the cell membranes; but polyhedra did not form in the whole infected cells. These results indicated that the *lacZ*-HcNPVs replicated like the wild-type virus in the insect cells [15].

The insertion of the *lacZ* gene sequence in the recombinant virus was confirmed by Southern hybridization analysis (Fig. 8). The *lacZ*-HcNPV recombinant DNAs, digested with *Bsu*36I or *Bgl*II, were hybridized with the *lacZ* gene probe. The *lacZ* gene probe was hybridized to the 18.5-kb fragment of *lacZ*-HcNPV DNA digested

with *Bgl*II (Fig. 8, lane 4) and the 12.25-kb fragments of *lacZ*-HcNPV genomic DNA doubly-digested with *Bsu*36I and *Bgl*II (Fig. 8, lane 5). These results indicated that the *lacZ* gene was inserted into the HcNPV genomic DNA.

Analysis of β-Galactosidase Production by the *lacZ*-HcNPV

The production of LacZ protein in *S. frugiperda* cells infected with the *lacZ*-HcNPV recombinant was detected by 10% SDS-PAGE (Fig. 9). The LacZ protein band, with a molecular mass of 116 kDa in the cells infected with the recombinant virus, appeared at 72 to 120 h on the polyacrylamide gel. There was no polyhedrin band (Fig. 9, lanes 3 and 5), but the cells infected with the wild-type of HcNPV formed a polyhedrin protein band on the gel with a molecular mass of 25 kDa [16] (Fig. 9, lane 2). These results indicated that the *lacZ*-HcNPV expressed the LacZ protein in the insect cell.

The production of the LacZ protein was also measured at time intervals by colorimetric assay using ONPG [20]. *S. frugiperda* cells infected with the *lacZ*-HcNPV recombinant were collected at time intervals, 24, 48, 72, 96, and 120 h p.i., and the activity of β-galactosidase was measured with a spectrophotometer at 420 nm. The enzyme activity appeared after about 24 h p.i. and increased continuously over time (Fig. 10). At 48 h p.i. the activity of the enzyme was about 50 IU/min/ml and at 120 h p.i. it was 170 IU/min/ml. This result indicates that the *lacZ* gene in the recombinant virus expressed LacZ protein under the control of the polyhedrin promoter in the cells.

When a foreign gene is expressed in the insect cell through the baculovirus expression system, the procedure is the same as the allelic replacement of the *lacZ* gene in the transfer vector into the wild-type virus.

This is the first report of research on the construction of a genetically engineered HcNPV. This research provides a very valuable host/vector system for many purposes.

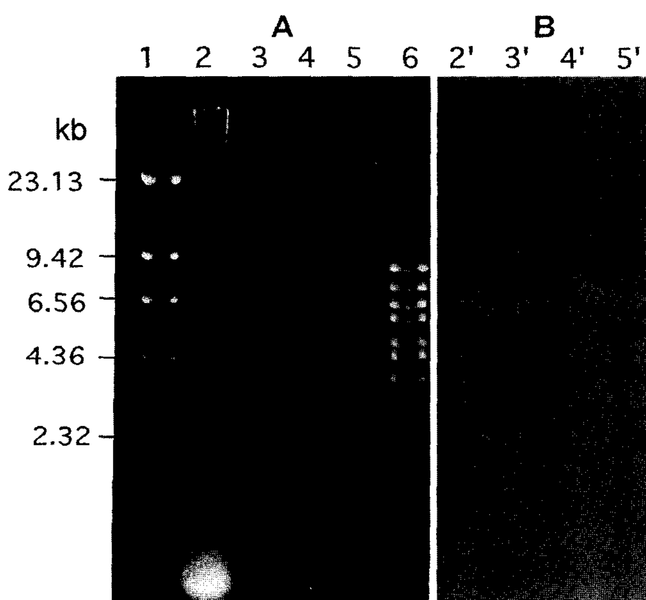

Fig. 8. Confirmation of the *lacZ* gene in *lacZ*-HcNPV recombinant by Southern hybridization analysis.

Lanes A1, λ phage DNA digested with *Hin*dIII; A2, the intact *lacZ*-HcNPV DNA; A3, A4, and A5, *lacZ*-HcNPV DNA digested with *Bsu*361, *Bgl*II, and *Bgl*II and *Bsu*361, respectively; A6, λ phage DNA digested with *Bst*EII. Panel B is the result of Southern blot of Panel A. B4' and B5' were hybridized with the *lacZ* gene probe.

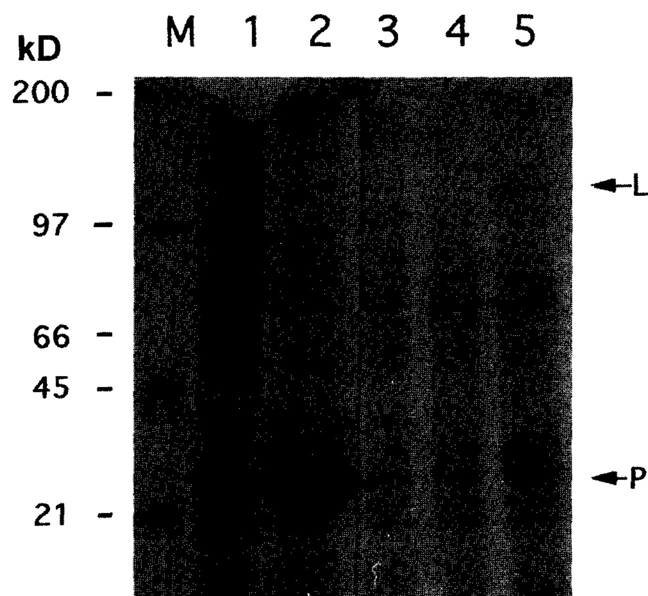

Fig. 9. Expression of β-galactosidase in the *lacZ*-HcNPV-infected *S. frugiperda* cells. Infected cell lysates were analyzed on a 10% SDS-PAGE at the time intervals.

Lanes M, molecular weight standards; 1, uninfected cells; 2, wild-type HcNPV-infected cells at 48 h; 3~5, *lacZ*-HcNPV-infected cells at 72, 96, and 120 h p.i. Abbreviations: L, LacZ protein; P, polyhedrin.

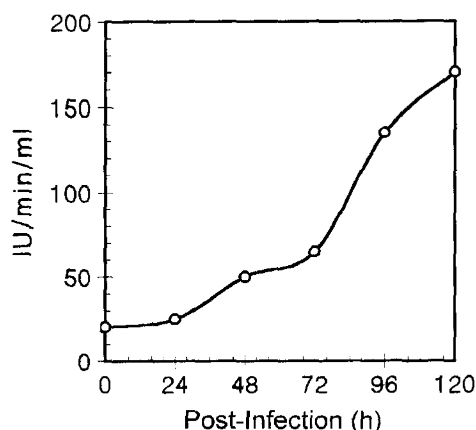

Fig. 10. Time course of β-galactosidase activity in *lacZ*-HcNPV-infected cells.

The enzyme activity was measured by colorimetric assay at 24, 48, 72, 96, and 120 h p.i.

This new baculovirus expression system may be of great value to those wishing to propagate and express large segments of eukaryotic foreign gene DNA in a eukaryotic environment.

Acknowledgments

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