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Construction of a Baculovirus *Hyphantria cunea* NPV Insecticide Containing the Insecticidal Protein Gene of *Bacillus thuringiensis* subsp. *kurstaki* HD1

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Abstract Baculovirus *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) insecticide containing the insecticidal protein (ICP) gene from *Bacillus thuringiensis* subsp. *kurstaki* HD1 was constructed using a *lacZ*-HcNPV system. The ICP (δ-endotoxin) gene was placed under the control of the polyhedrin gene promoter of the HcNPV. A polyhedrin-negative virus was derived and named ICP-HcNPV insecticide. Then, the insertion of the ICP gene in the ICP-HcNPV genome was confirmed by Southern hybridization analysis. Polyacrylamide gel electrophoresis (PAGE) analysis of the *Spodoptera frugiperda* cell extracts infected with the ICP-HcNPV showed that the ICP was expressed in the insect cells as 130 kDa at 5 days post-infection. The ICP produced in the cells was present in aggregates. When extracts from the cells infected with the ICP-HcNPV were fed to 20 *Bombyx mori* larvae, the following mortality rate was seen; 8 larvae at 1 h, 10 larvae at 3 h, and 20 larvae at 12 h. These data indicate that the *B. thuringiensis* ICP gene was expressed by the baculovirus insecticide in insect cells and there was a high insecticidal activity. The biological activities of the recombinant virus ICP-HcNPV were assessed in conventional bioassay tests by feeding virus particles and ICP to the insect larvae. The initial baculovirus insecticide ICP-HcNPV was developed in our laboratory and the significance of the genetically engineered virus insecticides is discussed.

Key words: Baculovirus, *Hyphantria cunea* NPV, δ-endotoxin, *Bacillus thuringiensis*, microbial pesticide

Baculoviruses possess a double-stranded, circular DNA genome with a molecular weight of approximately 8.7×10^7 [5, 15]. They are attractive as biological insecticides against certain invertebrates [4] and as vectors for propagating and expressing foreign genes in eukaryotic cells [12, 14, 21, 23] because they have extendable rod-shaped nucleocapsids [7, 18], circular DNA genomes [5], detectable non-essential polyhedrin genes [9, 13], and strong polyhedrin [22, 26] and p10 gene [24, 27] promoters.

Baculoviruses are safe to use as microbial insecticides because they are not harmful to mammals, birds, or plants. This limited host range makes baculovirus useful as insecticides [4]. On the other hand, baculovirus insecticides can take several days to kill the pest insects and their host ranges are not wide [6]. These undesirable aspects of baculoviruses limit their use in agriculture.

The development of baculoviruses as expression vectors of foreign genes has been reported with the use of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) [25, 31], and with the use of *Bombyx mori* NPV [23]. The authors have reported previously the development of a baculovirus expression system using HcNPV [14], which is a polyhedrin-negative virus system. In that research, the polyhedrin coding sequence was deleted and replaced with the *lac*Z gene because it does not affect virus replication [32] and, in addition, passenger DNAs may be propagated and expressed at a high level by the polyhedrin promoter [14, 22, 23, 25, 31].

A candidate gene for incorporation into a baculovirus insecticide is the insect-specific ICP (δ-endotoxin) gene from the *B. thuringiensis. B. thuringiensis* strains have shown lethalities against a wide range of insect species [4]. The presence of the active toxin within the gut of the insect causes an immediate reduction in feeding, which ultimately causes their death [8].

Previously, the authors have reported that the recombinant clone, pHLN1-80, containing the ICP gene of *B. thuringiensis* subsp. *kurstaki* HD1 was effective in killing insect larvae and encoded a 130-kDa protoxin [11], and

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a baculovirus expression system was also developed using HcNPV [14]. If the ICP gene in the recombinant plasmid were to be replaced into the *lacZ* gene site of the *lacZ*-HcNPV system, a strong and wide range of insecticides could be constructed. Therefore, we undertook this work with the goal of making a better baculovirus insecticide which has the ICP gene in its genome.

In this study, the insecticidal protein (ICP) gene of *B. thuringiensis* subsp. *kurstaki* HD1 was obtained from the plasmid pHLN1-80 and inserted into the genome of HcNPV to construct a recombinant baculovirus insecticide. A preliminary assessment of its effectiveness is described herein.

MATERIALS AND METHODS

Virus and Cell

The *lacZ-Hyphantria cunea* nuclear polyhedrosis virus (*lacZ*-HcNPV) strain-1 from this laboratory [20] was used for construction of an insecticide and propagated in *Spodoptera frugiperda* cells (IPLB-SF-21) [34] at 28°C in TC-100 medium (Gibco, Middletton, U.S.A.) supplemented with 5% fetal calf serum (Gibco, Middletton, U.S.A.), and then titrated for infectivity as described by Lee and Miller [18].

Bacteria and Plasmids

Escherichia coli XL1-Blue/pHLN1-80 clone and *Escherichia coli* XL1-Blue/pHLN2-80 clone from this laboratory [11], which contain a full-length ICP gene from *Bacillus thuringiensis* subsp. *kurstaki* strain HD1, was used for the ICP gene source. *Escherichia coli* XL1-Blue/pBluescript SK(+) [30] and pBacPAK9 [12] were used for cloning.

Silkworm

Silkworms (*Bombyx mori* larvae) used for bioassay were obtained from Dr. S. P. Lee (National Institute of Agricultural Science and Technology, Suwon, Korea).

Oligonucleotide and Probe

Oligonucleotide sequences (primer) for PCR were synthesized from Korea Biotech Inc., Taejon, Korea (Table 1). Primer A contained the *Nde*I site and translation start codon ATG, and primer B contained the *Xho*I site and translation termination condon TAG. The probe to identify the ICP gene was pHL-0.9 plasmid containing part of the ICP gene of *B. thuringiensis* made in this laboratory.

Table 1. List of primers used for PCR in this study.

Primer	Nucleotide sequences (5'→3')
A	5'-AATTGGATACAT**ATG**GACAACAACCCGAAC-3'
B	5'-CCCCCTCGAG**CTA**GTTAATATGATAATCCG-3'

The *Nde*I site is underlined and the ATG translation start codon is boldfaced in primer A. The *Xho*I site is underlined and nucleotide sequences complementary to the TAG termination codon is boldfaced in primer B.

Virus Purification

Viral multiplication and nonoccluded virus purification were carried out with the procedure described by Lee *et al.* [16] and Lee and Lee [17].

Plasmid Isolation

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. Then, the plasmid DNA was purified by the Birnboim and Doly [1] procedure.

Restriction Enzyme Digestions and Agarose Gel Electrophoresis

All restriction endonuclease digestions were performed according to the manufacturer's instructions. HcNPV genomic DNA and vector DNAs were digested and electrophoresed on 1.0% agarose gel, and the molecular sizes of each DNA fragment were determined by comparing their mobility with *Hin*dIII-digested phage λ DNA fragments. The reactions were ended by the addition of 1/10 volume of a stop solution (7 M urea, 50% sucrose, 0.1 mM EDTA, 0.1% bromophenol blue). Details of gel electrophoresis and visualization of the DNA fragments have been described by Lee *et al.* [15].

Cloning and Transformation

Cloning was carried out by mixing together 15 μl (0.2 μg) of inserting 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 reacting 50 μl of the solution for 18 h at 14°C [20]. The reaction condition was examined by 1.0% agarose gel electrophoresis [15]. The *E. coli* competent cells were prepared and transformed by the Mandel and Higa method [19].

Purification of DNA from Agarose Gels

Restriction DNA fragments were fractionated in 1.0% low-melting-temperature agarose gels containing Tris-borate and ethidium bromide. The bands were identified using 300 nm long wavelength ultraviolet light and then excised from the gel. The sliced gel was melted at 65°C, diluted to 0.2% agarose in 100 mM Tris-HCl (pH 8.0), cooled to 37°C, and mixed with phenol for 25 min at 37°C. The organic and aqueous layer was reextracted briefly with phenol/chloroform (1:1) in a TE buffer (10 mM Tris-Cl, 1.0 mM EDTA). After recentrifugation at 10,000×g, the DNA pellets were precipitated with ethanol, repelleted, and dissolved in the TE buffer.

Construction of Transfer Vectors Containing the ICP Gene

Construction of pHL-1.9. The plasmid pHLN1-80, containing the ICP gene from *B. thuringiensis* subspecies *kurstaki* strain HD1, was obtained from this laboratory [11]. A part of the ICP gene of the plasmid was amplified using primers A and B by a polymerase chain reaction [28] to generate *Nde*I and *Xho*I sites. The 1.9-kb PCR product was treated with Klenow fragment to make blunt ends. Then it was digested with *Xho*I, inserted into the *Sma*I and *Xho*I sites of pBluescript SK(+), and named pHL-1.9 recombinant clone (Fig. 1).

Construction of pHL-3.7. Digestion of the pHLN1-80 with *Sac*I enzyme generated 5.4-kb and 1.3-kb DNA fragments. The 1.3-kb *Sac*I-*Sac*I the ICP gene at the 5' end was removed (Fig. 1). Also, the pHL-1.9 plasmid DNA was digested with *Sac*I enzyme to generate a 1.3-kb DNA fragment containing part of the ICP gene. The fragment was purified from a low-meeting-temperature agarose gel [15] and inserted into the *Sac*I site of the

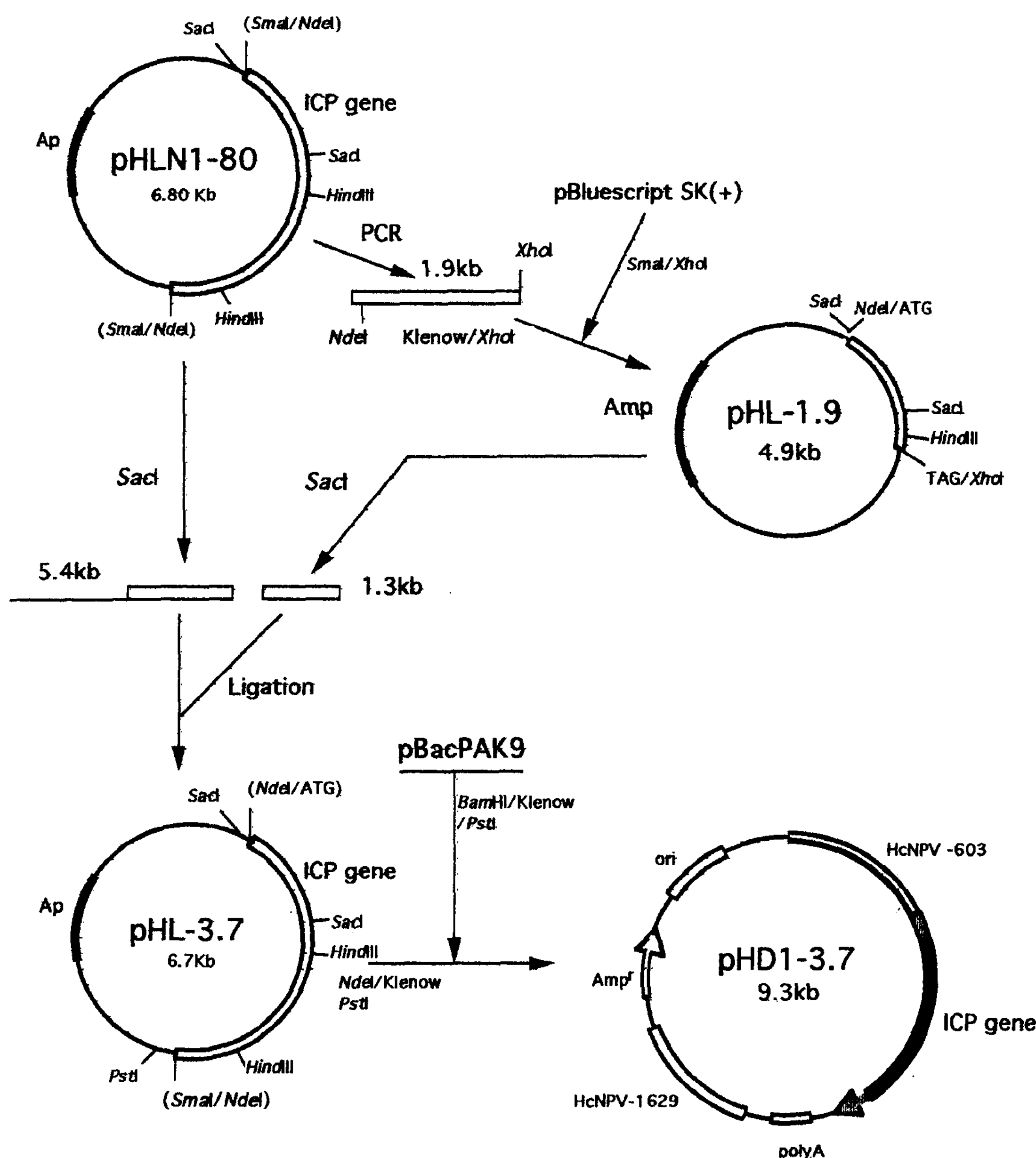

Fig. 1. Construction scheme for pHD1-3.7 clone.

A 1.9-kb fragment containing *Nde*I and *Xho*I sites from the pHLN1-80 by PCR was created, cloned into pBluescript SK(+), and named pHL-1.9. The pHLN1-80 and the pHL-1.9 clones were digested with *Sac*I and ligated to construct pHL-3.7 recombinant. The ICP gene in the pHL-3.7 was digested out with *Nde*I, treated with Klenow fragment, digested with *Pst*I, then cloned into the *Pst*I site of pBacPAK9, and named pHD1-3.7.

5.4-kb linearized pHLN1-80 plasmid to generate pHL-3.7 containing a full length of the ICP gene.

Construction of pHD1-3.7. The 3.7-kb ICP gene sequence was digested with *Nde*I, treated with Klenow fragment, digested with *Pst*I enzyme and then purified in the agarose gel. Baculovirus transfer vector pBacPAK9 DNA was digested with *Bam*HI, treated with Klenow fragment to make blunt ends, and digested with *Pst*I enzyme. Then, the 3.7-kb ICP sequence was inserted into the *Pst*I site of the vector and named pHD1-3.7 (9.3 kb) (Fig. 1).

Construction of Recombinant Virus Insecticides

The *S. frugiperda* cells were cotransfected with *lacZ*-HcNPV DNA mixed with pHD1-3.7 plasmid DNA and putative recombinants ICP-HcNPVs lacking polyhedra were isolated (Fig. 3) as previously described by Lee *et al.* [14, 16].

Selection of Recombinant Viruses by Plaque Assay

Plaque assay was performed using a modification of procedures described previously [14, 16, 18] to select recombinant viruses that contain the ICP gene in their genomic DNAs. The cotransfection supernatant was used as original inoculum for the plaque assay and inoculated into *S. frugiperda* cell monolayers in dishes (60×15 mm). The method utilizes 1.5% low-melting point agarose (Sea Kem) and X-gal (240 μg/ml) to screen the β-galactosidase gene in supplemented TC-100 medium as an overlay. Recombinant virus plaque, which produces white plaque, was picked from each plate.

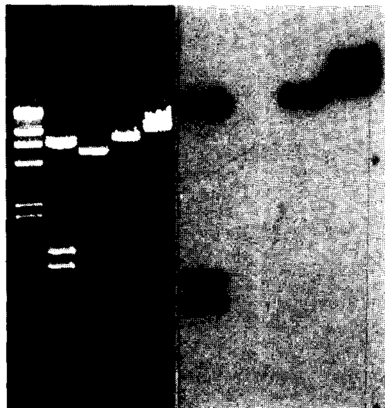

Fig. 2. Confirmation of insertion of ICP gene in the pHL-1.9 and pHD1-3.7 clones.

Panel A is the 0.6% agarose gel electrophoregram and panel B is the Southern blot of the A gel. Lanes 1, λ DNA digested with *Hin*dIII; 2, pHD1-3.7 DNA digested with *Hin*dIII; 3, pBacPAK9 DNA digested with *Not*I; 4, pHL-1.9 DNA digested with *Not*I; and 5, pHD1-3.7 DNA digested with *Not*I.

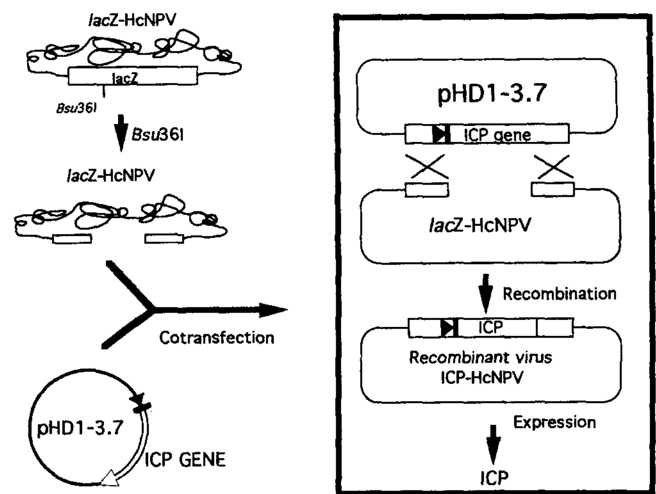

Fig. 3. Construction scheme for a recombinant virus ICP-HcNPV.

The *lacZ*-HcNPV was digested with *Bsu*361 enzyme and then cotransfected with pHD1-3.7 clone DNA into *S. frugiperda* cell to make the recombinant virus.

There were four passages of the virus through the *S. frugiperda* cell.

SDS-PAGE Analysis and Southern Hybridization

ICP extracts were fractionated on 10% polyacrylamide gels as described by Bollag *et al.* [3]. Southern hybridization analysis was performed using the procedure described by Southern [33].

Bioassay

Bombyx mori larvae were maintained in the laboratory on a semi-synthetic diet (National Institute of Agricultural Science and Technology, Suwon, Korea). *S. frugiperda* cells were infected with ICP-HcNPV insecticide (2 pfu (plaque forming unit/cell), cultured for 5 days, and harvested at 5,000×g for 20 min. The supernatants were discarded and the pellets were resuspended in 2 ml of phosphate-saline buffer (0.268 g of $Na_2HPO_4 \cdot 7H_2O$, 1.425 g of KH_2PO_4, 8.2 g of NaCl, and 3.0 g of KCl in 1.0 liter of H_2O, pH 6.2) [18], washed once, and resuspended in 2.0 ml of H_2O. The resuspended solution (0.2 ml) was applied uniformly to the surface of a 2×2 cm^3 synthetic diet in each dish. Twenty *B. mori* 3rd instar larvae were introduced in two dishes and fed the infected diets. One dish was for a control. Larvae were starved for 4 h before treatment. The 0.1 ml treatment dosages each had 2.0×10^5 cells containing the ICP. Larvae were maintained at room temperature, constant light, and 40% humidity. Additional food (untreated) was presented to the larvae upon consumption of the diet in order to avoid starvation effects. Mortality was monitored for 24 h after exposure to the treatment diets. Lethality was calculated by counting the dead.

RESULTS

Construction of a Recombinant Insecticide, ICP-HcNPV

The ICP gene from *B. thuringiensis* subsp. *kurstaki* strain HD1 was cloned into the baculovirus transfer vector and then recombinated into the *lacZ-Hyphantria cunea* nuclear polyhedrosis virus expression vector (Fig. 1). The ICP coding sequences were amplified from pHLN1-80 clone with PCR and inserted into pBluescript SK(+) to produce pHL-1.9, which was digested with *Sac*I enzyme to generate the 1.3-kb part of the ICP coding sequences. The 1.3-kb sequences were also inserted into the *Sac*I site of the pHLN1-80 plasmid to produce a pHL-3.7 clone. The 3.7-kb ICP gene of the pHL-3.7 clone was transferred into the baculovirus transfer vector pBacPAK9 and named pHD1 (Fig. 1). The ICP gene sequences in the clones were confirmed by Southern hybridization analyses (Fig. 2). Figure 2 indicates that those clones contained the ICP gene sequences.

The transfer vector pHD1-3.7 DNA was cotransfected with infectious *lacZ*-HcNPV DNA to produce a polyhedrin-negative mutant, ICP-HcNPV, with the ICP gene replacing the *lacZ* gene (Fig. 3). The ICP-HcNPV recombinant insecticide was isolated by plaque assay (Fig. 4). These were plaque-purified to genetic homogeneity and further multiplied to provide stocks of the virus with titres similar to those of normal HcNPV (10^7 to 10^8 pfu/ml). Southern hybridization analysis of DNA extracted from cells infected with the ICP-HcNPV confirmed the insertion of the ICP gene in the 4.47-kb *Eco*RI fragment (Fig. 5).

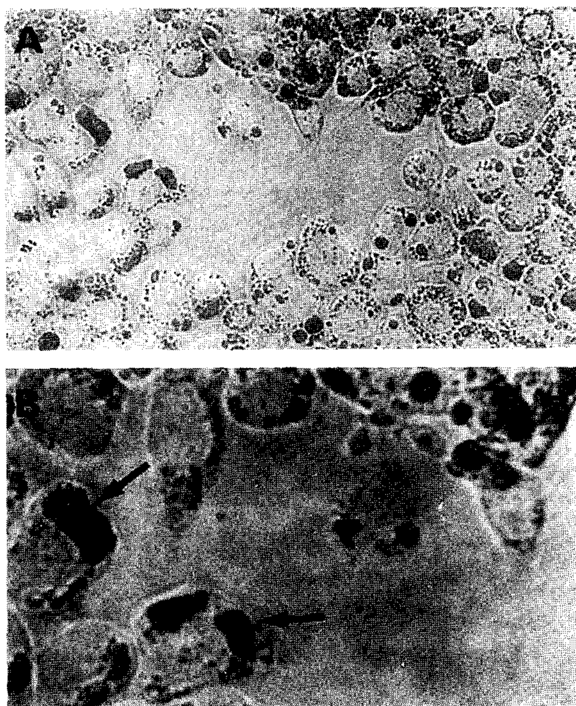

Fig. 4. Micrographs of plaques formed by the recombinant virus ICP-HcNPV on *S. frugiperda* cell monolayer.

A is a plaque formed by infection of the ICP-HcNPV (×200). B is the magnified micrograph of the A (×400). The cells did not form polyhedra in the infected cells; however, the ICP may be aggregated in the cells. Arrows indicate ICP aggregated in the cells infected with the viruses.

Expression of the *B. thuringiensis* ICP in Insect Cells

Mock-infected *S. frugiperda* cells, *S. frugiperda* cells infected with the ICP-HcNPV, and *E. coli* bearing with either the pHLN1-80 clone [11] or the pHLN2-80 clone[11] were analyzed using a 10% PAGE (Fig. 6). These results showed that 130 kDa polypeptide bands appeared at lanes 4 to 7 (Fig. 6). Lanes 2 and 3 are negative controls, and lanes 4-6 are positive controls for comparison with the ICP-HcNPV clone. Lane 4 is the *kurstaki* HD1 lysate, wherein the crystal ICP was not appeared due to denaturation. Lane 5 is the lysate of the *E. coli* containing the pHLN1-80 clone. Lane 6 is the lysate of the *E. coli* bearing pHLN2-80. Lane 7 is the lysate of the *S. frugiperda* cells infected with the ICP-HcNPV recombinant virus. The electrophoregram (Fig. 6, lane 7) demonstrates that the putative ICP was produced most prolifically in *S. frugiperda* cells infected with the ICP-HcNPV recombinant virus at about 5 days p.i. Figure 4 shows that no polyhedra were formed in the cells, and that the cells were hypertrophied. High amounts of the putative ICP were found to be aggregated in the cells.

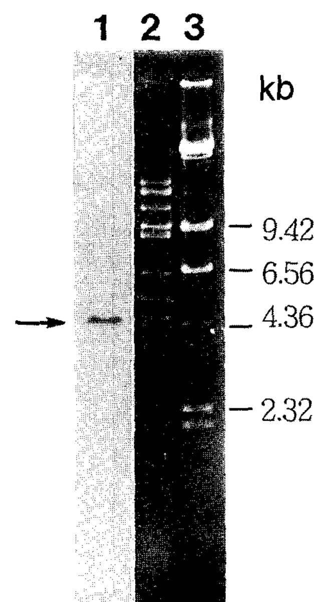

Fig. 5. Detection of ICP gene insertion in ICP-HcNPV recombinant genome.

The ICP-HcNPV genome DNA was digested with *Eco*RI enzyme. Southern hybridization analysis of the gel was performed with the probe to detect the ICP gene insertion. Lanes 1, Southern blot of the gel; 2, ICP-HcNPV DNA digested with *Eco*RI; and 3, λ DNA digested with *Hin*dIII. Arrow indicates the ICP gene-inserted fragment band.

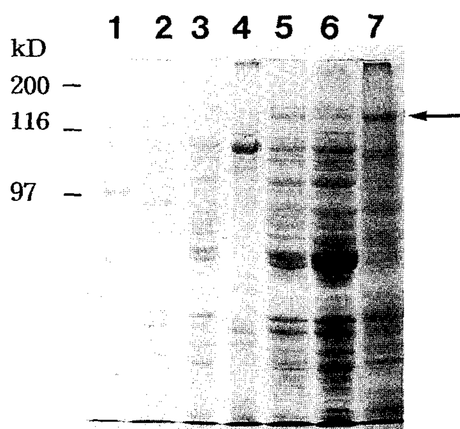

Fig. 6. Detection of ICP produced in *S. frugiperda* cells by ICP-HcNPV virus.

The production of ICP in the cells infected with the recombinant virus was detected on 10% SDS-PAGE analysis. Lanes 1, standard molecular markers; 2, *E. coli*/pBluescript SK(+) lysate; 3, *S. frugiperda* cell lysate; 4, lysate of *Bt kurstaki* HD1 culture; 5, *E. coli*/pHLN1-80 lysate; 6, *E. coli*/pHLN2-80 lysate; and 7, lysate of *S. frugiperda* cells/ICP-HcNPV. Arrow indicates 130 kDa ICP.

Table 2. Lethalities of ICP produced in *S. frugiperda* cells by ICP-HcNPV infection against *B. mori* 3rd larvae

time	Lethalities at the following time (h)							
	0	1	2	3	4	7	9	12
killed	0	8	9	10	13	15	19	20
lived	20	12	11	10	7	5	1	0

Lethality of Cell Lysate Infected with ICP-HcNPV

The *in vivo* activity of the ICP was assessed by feeding extracts from cells infected with the ICP-HcNPV to 3rd instar *Bombyx mori* larvae. Uninfected cells were used as controls. The usual protocol with this bioassay is to feed the samples to insects on a small lump of semi-synthetic diet. Insects were fed the equivalent of 5,000 cells infected with ICP-HcNPV. When the biological activity of ICP-HcNPV virus was assessed, feeding was markedly inhibited. When extracts from the cells infected with the ICP-HcNPV were fed to twenty *B. mori* larvae, the following mortality rate was seen; 8 larvae at 1 h, 10 larvae at 3 h, and 20 larvae at 12 h (Table 2).

DISCUSSION

B. thuringiensis subsp. *kurstaki* HD1 produces an insecticidal protein (ICP) of about 130 kDa, otherwise known as the δ-endotoxin [11, 29], which kills certain insect larvae [10]. The ICP gene cloned into the baculovirus transfer vector (Fig. 1) was replaced by the *lacZ* gene in the *lacZ*-HcNPV vector and named ICP-HcNPV recombinant virus (Fig. 3). The replacement derives a polyhedrin-negative recombinant virus where expression of the ICP gene was mediated by the polyhedrin promoter. The putative ICP proteins were aggregated in the cells infected with the virus (Fig. 4B) and confirmed by SDS-PAGE analysis (Fig. 6, lane 7). A similar construction was reported by Merryweather *et al.* [21], wherein they cloned *B. thuringiensis* subsp. *kurstaki* HD-73 endotoxin (ICP) gene in *Autographa californica* nuclear polyhedrosis virus. In that research, they used strains of bacterium and insect virus systems different from the ones investigated here. They constructed a recombinant virus insecticide based on AcMNPV, which was toxic to *Trichoplusia ni* larvae; however, they did not find ICP proteins aggregated in cells.

Biological activity of the ICP was determined by bioassays. The insect larvae refused to consume a diet contaminated with infected cell extracts. Therefore, larvae were starved for 4 h before treatment. The presence of ICP subsequent to feeding inhibition is a characteristic of insects that ingest the natural *B. thuringiensis* product [8]. These data indicate that the ICP protein produced by the insect cells is biologically active (Table 2). Cleavages of the ICP (endotoxin) usually occur in the gut of the caterpillar [22].

The use of genetically engineered viruses was investigated in a regulated environment [2]. However, infected insects which produce active endotoxin material will permit the effect on other insects to be controlled. Therefore, the efficacy of these viruses as genetically engineered biological control agents should not be judged solely on the basis of preliminary laboratory studies. This first report of research on the construction of a genetically engineered HcNPV containing *B. thuringiensis* endotoxin gene will guide further research on developing new insecticides based on the *lacZ*-HcNPV vector system.

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