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Short communication

Cloning, Sequencing and Baculovirus-based Expression of Fusion-Glycoprotein D Gene of Herpes Simplex Virus Type 1 (F)

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The Glycoprotein D (*gD*) gene of the HSV-1 strain F was cloned, sequenced, recombinated into the HcNPV (*Hyphantria cunea* nuclear polyhedrosis virus) expression vector and expressed in insect cells. The *gD* gene was located in the 6.43 kb *Bam*HI fragment of the strainF. The open reading frame (ORF) of the *gD* gene was 1,185 bp and codes 394 amino acid residues. Recombinant baculoviruses, GD-HcNPVs, expressing the gD protein were constructed. *Spodoptera frugiperda* cells, infected with the recombinant virus, synthesized a matured gX-gD fusion protein with an approximate molecular weight of 54 kDa and secreted the gD proteins into the culture media by an immunoprecipitation assay. The fusion gD protein was localized on the membrane of the insect cells, seen by using an immunofluorescence assay. The deduced amino acid sequence presents additional characteristics compatible with the structure of a viral glycoprotein: signal peptide, putative glycosylation sites and a long C-terminal transmembrane sequence. These results indicate the utility of the HcNPV-insect cell system for producing and characterizing eukaryotic proteins.

Keywords: HSV-1, Glycoproteins, Baculovirus; Glycosylation, gX signal sequence.

Introduction

The Glycoprotein D of the *Herpes simplex* virus (HSV) is a component of the virion envelope. Its gD plays an important role in viral adsorption in cells, penetration into cells, and cell fusion (Fuller and Spear, 1985; Highlander *et al.*, 1987; Johnson and Ligas, 1988). HSV-1 gD is a dominant antigen for both humoral and cell-mediated immune responses to HSV-1 infection (Blacklaws *et al.*, 1987; Martin and Rouse, 1987; Krishna *et al.*, 1989). Prophylactic immunization of naive animals with gD has provided protection against primary infection and reduced latency (Wachsman *et al.*, 1989). As such, gD is one of the principal antigens for a HSV subunit vaccine. Watson *et al.*, (1982) determined the nucleotide sequences of the gD gene of the HSV-1 strain and expressed it using the *E. coli* system. A number of recombinant systems have been used in the production of gD. These include the bacterial system (Watson *et al.*, 1982), vaccinia virus (Wachsman *et al.*, 1989), and baculovirus expression systems using the *Autographa californica* nuclear polyhedrosis virus (AcNPV) (Krishna *et al.*, 1989; Sisk *et al.*, 1994). Production systems have shown varying degrees on immunogenicity in animal models. Baculovirus expression vectors have proven useful for achieving a high-level expression of a variety of foreign genes, and for obtaining biologically active polypeptides in insect cells (Smith *et al.*, 1983; O'Reilly and Miller, 1994). We previously constructed a baculovirus transfer and expression vector based on HcNPV (Lee, 1987; Lee and Lee, 1988) in order to achieve a high level of expression of a variety of foreign genes in *Spodoptera frugiperda* cells (Lee *et al.*, 1998a, 1998b, 1999). Specifically, the current study was designed to study cloning, sequence analysis, expression, and properties of gD of the HSV-1 strain F (Ejercito *et al.*, 1968), which is a mild pathogen for humans. Accordingly, using these systems, the gD coding sequence of HSV-1 (F) was recombined into the HcNPV expression vector. The resulting recombinant gD protein was then characterized.

Materials and Methods

Viruses and cells The *Herpes simplex* virus type 1 (HSV-1) strain F (ATCC VR-733) was obtained from the Korean AIDS (Acquired Immune Deficiency Syndrome) Center, Seoul, Korea. The Vero cell (ATCC CCL81) was obtained from the Korean Type Culture

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Collection (KTCC). The HSV-1 strain was grown at 37°C in the Vero cells using Eagle's minimum essential medium (Gibco, Detroit, MI,USA) with 10% fetal bovine serum (Gibco), as described by Kang *et al.*, (1996) and Cho *et al.*, (2000). The *lacZ-Hyphantria cunea* nuclear polyhedrosis virus (*lacZ*-HcNPV) lacking the polyhedrin gene (Lee *et al.*, 1998a) was propagated in *Spodoptera frugiperda* cells (IPLB-SF-21) (L. K. Miller, University of Georgia, USA) at 27°C in a TC-100 medium (Gibco, Middleton, WI, USA). The cells were routinely maintained in a TC-100 medium (Gibco, Gaithersburg, MD, USA) that was supplemented with 0.2% tryptose broth powder and 10% fetal bovine serum, and passaged every 5 days. The virus inoculum used was an extracellular nonoccluded virus (NOV) derived from a cell culture medium, and titered by a plaque assay (Lee and Miller, 1978).

Bacterial strains and plasmids The pBacPAK9 (5.5 kb) (Clontech Co. Palo Alto, CA, USA) and pHcgXIIIB plasmid (6.7 kb) harbored in *E. coli* XL1-Blue (Lee *et al.*, 1999) were used for the HSV-1 *gD* gene cloning and transfer into the expression viral vector (*lacZ*-HcNPV) (Lee *et al.*, 1998a). A pBluescript SK (+) plasmid (2.96 kb) (Stratagene, La Jolla, CA, USA) was used for both the cloning and sequencing. The pHLA-17 plasmid (9.3 kb) harbored in *E. coli* XL1-Blue (Kang *et al.*, 1996) was used for the HSV-1 *gD* gene source.

Oligonucleotides The oligonucleotide primers (A-1, 5' : 5'-GGG AGATCT**ATG**TGTGGTGCGTTCCGGTAT-3' and B-1, 3' : 5'-CCCGAATTC**CTA**CCCCCCCGCACCCATTAA-3') and a probe (P-1, 5'-GTGCGTTCCATGGGGGGGACTGCCGCCAGGTTG-3') were deduced from the terminal sequences of the published sequences of the *gD* gene of HSV-1 (Watson *et al.*, 1982), and used for the PCR and colony hybridization, respectively. The primer A-1 contained the *Bgl*II site (underlined) and a translation start codon ATG (bolded); the primer B-1 contained the *Eco*RI site (underlined) and a termination codon TAG (bolded). The primers (Hc-1, 3 : 5'-TGTTAACCTTCTCCC-3' and Hc-2, 5 : 5'-CACGTCGAGTCAA TTGTAC-3') were deduced from the terminal sequence of the published sequence of the polyhedrin gene of HcNPV (Lee *et al.*, 1992) and used for the partial sequencing of the gene inserted in the HcNPV transfer vector and a PCR of the recombinant viruses. The oligonucleotides were synthesized by the Korea Biotec Inc., Daejeon, Korea.

Monoclonal antibody The HSV-1 strain F gD-specific monoclonal antibody (Biodesign International, Saco, Maine, USA) and the fluorescein isothiocyanate- conjugated sheep anti-mouse IgG were purchased from Boeringer Mannheim Biochemicals (Indianapolis, IN, USA).

Preparations of DNAs *E. coli* containing the recombinant plasmids was cultured in a LB broth (Difco Laboratories, Detroit, MI, USA) with proper antibiotics at 37°C. The plasmid DNA was purified according to the procedure described by Birnboim and Doly (1979). The *lacZ*-HcNPV DNAs were purified based on the procedure described by Lee *et al.* (1998a), and Lee and Kim (1999).

Colony and Southern blot hybridizations A colony hybridization for the detection of colonies containing *gD* gene fragments of the HSV-1 *Bam*HI genome library (Kang *et al.*, 1996) was carried out according to the procedure described by Grunstein and Hogness (1975) using the probe P-1. The *gD* gene locus in the selected recombinant plasmids was confirmed by a Southern blot analysis (Southern, 1975).

Amplification of DNAs DNAs were amplified by a polymerase chain reaction (PCR) according to the procedure described by Chung *et al.* (1994). The PCR analysis was conducted with 1.0 mg of the template DNA, 0.25 mg each primer, 200 mM deoxynucleoside triphosphates and 2 ml DMSO. The mixture solution was heated to 100 for 7 min, put on ice for 10 min, then 1 unit of Taq polymerase was added. Thirty cycles of amplification were performed and the last cycle was passed on a thermocycler (Perkin-Elmer). Aliquots of the amplification products (1/10 volume) were analyzed on 0.8% agarose gels.

Restriction enzyme digestions and agarose gel electrophoresis All restriction endonuclease digestions were performed according to the manufacturer's instructions. The DNAs were digested and electrophoresed on a 1.0% agarose gel. The details of the gel electrophoresis and visualization of the DNA fragments were previously described by Lee and Miller (1978) and Sambrook *et al.* (1989).

Cloning and transformation The HSV-1 *gD* gene in the plasmid pHLA-17 was amplified by PCR and a recombinant plasmid, pHcGD, was constructed using standard protocols (Sambrook *et al.*, 1989; Lee *et al.*, 1998a,b), as described in Fig. 1A. The plasmid pHLA-17 was amplified with the two primers containing the *Bgl*II, or the *Eco*RI site at the ends to create a 1.2 kb of the *gD* gene sequence. This gene fragment was then inserted into the *Bgl*II and *Eco*RI sites of the baculovirus transfer vector pBacPAK9, then the resulting plasmid was named pBac-gD. This *gD* gene was digested out with the *Bgl*II and *Bam*HI enzymes, and cloned into the *Bam*HI site of the pHcgXIIIB transfer vector. The resulting recombinant was named pHcGD plasmid (Fig. 1A). Competent *E. coli* cells were prepared and transformed by the procedure described by Mandel and Higa (1970).

Determination of nucleotide sequences The *gD* gene in the pBac-gD clone (Fig. 1A) was digested with the *Bgl*II and *Bam*HI enzymes, and cloned into the *Bam*HI site of pBluescript SK (+) and named pB-gD (Fig. 2, lane 7). The *gD* gene in the pB-gD plasmid was digested with the *Sac*II or *Apa*I, and the resulting fragments were ligated into the *Bam*HI site of the pBluescript SK (+) vector. The resulting recombinant plasmids were sequenced using the dideoxynucleotide chain termination procedure (Sanger *et al.*, 1977) on a polyacrylamide sequencing gel using approximately 8 μg/μl of double-stranded DNA templates and 100 pmol/μl of a limiting primer. The DNA fragments were labeled with ^{35}S-ATP (Amersham-pharmacia, Buckinghamshire, UK) and sequenced with a 7-deaza-dGTP sequencing kit that included the Sequenase version 2.0 T7 DNA polymerase (United States Biochemical, Cleveland, OH, USA) using a pBluescript T3 primer, T7 DNA primer, and M13 reverse primer.

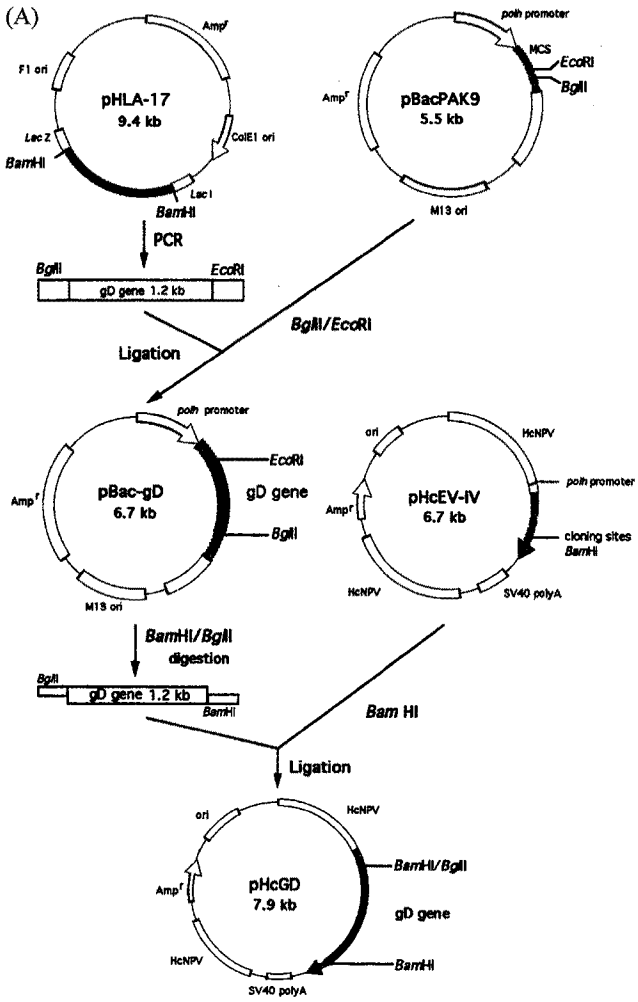

Fig. 1. Construction scheme for pHcGD plasmid containing the HSV-1 *gD* gene sequence. (A) A 1.2 kb gB gene sequence in the plasmid pHLA-17 was amplified with PCR, which was cloned into the pBac-PAK9 transfer vector to construct the pBac-GD recombinant plasmid. Then the *gD* gene was transferred into the pHcgXIIIB transfer vector to generate a pHcGD plasmid. (B): Partial nucleotide sequence of the cloning site downstream of the polyhedrin promoter region. Part of the downstream of the promoter, the ligated *Bam*HI/*Bgl*II site, and the translation start codon ATG of the *gD* gene, were all apparent.

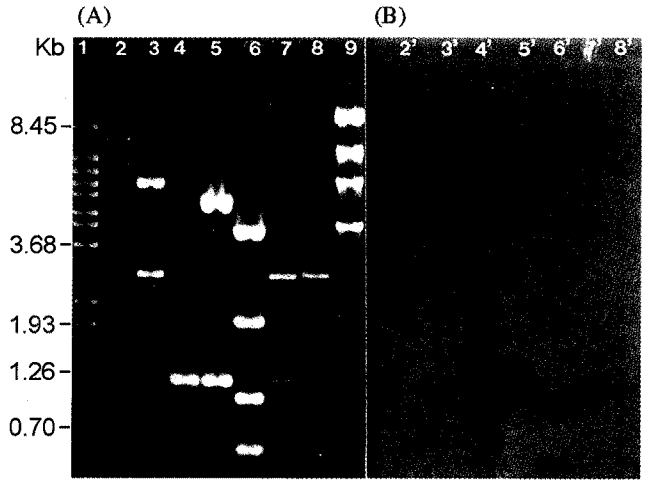

Fig. 2. Agarose gel analysis of *gD* gene fragments (A) and identification of *gD* gene fragments in the recombinant clones by Southern blot analysis (B). The digested DNAs were electrophoresed for 17 h on a 1.0% agarose gel. Lanes 1, DNAs digested with *Bst*EI; 2, HSV-1 genomic DNAs digested with *Bam*HI; 3, pHLA-17 plasmid DNAs digested with *Bam*HI; 4, PCR DNAs of *gD* gene; 5, pBac-GD plasmid DNAs double-digested with *Bgl*II plus *Eco*RI; 6, pHcGD(+) plasmid DNAs with *Bam*HI; 7, pB-gD DNA digested with *Bam*HI plus *Xba*I; 8, pB-gD DNAs digested with *Eco*RI; 9, λ phage DNAs digested with *Hind*III. The probes used were the *Asp*I 3.1 kb fragment containing the *gD* gene sequence.

Construction and selection of recombinant baculoviruses The *S. frugiperda* cells were seeded in 60 mm petri dishes with 2×10^6 cells per dish and cotransfected with two microliters (1.0 μg of DNA) of linearized *lac*Z-HcNPV DNA with the *Bsu*361 enzyme and 10 μl (10 μg) of pHcGD plasmid DNA in a TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH7.5) in 40 μl of H_2O to construct recombinant viruses using lipofectin-transfection (Lee *et al.*, 1998a, 1998b). The recombinant viruses in the supernatants were selected using a 1.5% low-melting-point agarose plaque assay (Lee *et al.*, 1998a) containing X-Gluc, and then incubated at 27°C for 5 days. The blue occlusion-negative plaques were picked and added to a serum-free medium. The insertion of the *gD* gene in the recombinant virus was analyzed by PCR using the primers Hc-1 and Hc-2, and a Southern blot (Southern, 1975).

SDS-PAGE for detection of gD protein The gD proteins were electrophoesed on 10% polyacrylamide gels (Bollag *et al.*, 1996). The *S. frugiperda* cells (1.5×10^7 cells per flask) were seeded in a flask (75 cm^2), allowed to attach in a mono-layer overnight at 27°C, infected with the recombinant baculoviruses expressing the gD at a multiplicity of infection of 2 pfu/cell, and incubated with 4 ml of a TC-100 medium for 96 h at 27°C. The infected cells and media at 96 h pi were separated by centrifugation at $12,000 \times g$ for 5 min at 4°C. The cells were resuspended in 5 ml of PBS (pH 6.2), freeze-thawed, and dialyzed to be a final concentration of 1.0 ml. The media were dialyzed to the final concentration. Normal *S. frugiperda* cells, and cells infected with the wild-type *lac*Z-HcNPV as a control, were treated in the same manner as the infected cells.

Radioimmunoprecipitation assay The gD proteins were identified using a radioimmunoprecipitation assay described by Cha *et al.* (2000). The *S. frugiperda* cells (1×10^6 cells per dish) were seeded on 35 mm petri dishes and allowed to attach in a monolayer overnight at 27°C. The cells were each infected with the glycoprotein gene recombinant virus at a multiplicity of infection of 2 pfu/cell. After 1 h of adsorption at 27°C, the monolayers were washed with PBS (pH 6.2) (Lee and Miller, 1978), and the cells were incubated with 2 ml of a TC-100 medium for 48 h at 27°C. After incubation, the monolayers were washed twice in a prewarmed leucine-deficient TC-100 basal medium (Sigma), and

incubated in 0.5 ml of a leucine-deficient basal medium (Sigma) for 1 h at 27°C. The medium was then removed and replaced with 0.5 ml of a leucine-deficient basal medium containing 20 μCi [^{3}H]-leucine (Amersham-pharmacia Biotech). The cells were incubated for 1 h at 27°C with gentle rocking. After labeling, the cells were lysed in an immunoprecipitation buffer (Cha *et al.*, 2000) and incubated with the gD specific monoclonal antibody for the antigen-antibody complex formation. Thereafter, the immunoprecipitation products were separated on a 10% SDS-polyacrylamide gel. The gel was then dried, exposed to film, and developed for 3 min at 20°C in a developer.

Immunofluorescence assay An immunofluorescence assay (IFA) for detecting the gD proteins in cells was carried out using the procedures described by Ghiasi *et al.* (1991) and Lee *et al.* (1999). The *S. frugiperda* cells (2×10^6 cells per dish) were seeded on a glass cover slip (18×18 mm) in 60 mm dishes, infected with the recombinant baculoviruses expressing gD at a multiplicity of infection of 10 pfu/cell, and incubated for 72 h. The infected cells grown on the glass cover slip were fixed with acetone for 15 min. The fixed cells were then washed in PBS (pH 6.2) for 10 min and incubated for 1 h at room temperature with a gD-specific monoclonal antibody diluted in a 0.5% BSA solution in PBS at 1 : 500 and 1 : 2, respectively. The cells were rinsed three times for 5 min with PBS, then incubated for 1 h with an anti-mouse immunoglobulin fluorescein (Boehringer Mannheim) diluted 1 : 50 with a 0.5% BSA solution. The cells were washed twice for 5 min with PBS, mounted in a mounting buffer (1 glycerol : 5 PBS), then examined through a fluorescent microscope (AO Fluoro-star 20, USA).

Results and Discussion

Construction of a GD-HcNPV recombinant virus expressing the gD protein A *Bam*HI fragment library of the HSV-1 strain F DNA genome was screened with the P-1 probe. A number of positive colonies were identified. One colony, which strongly hybridized with this probe, was isolated. The purified DNAs of the clone were amplified by a PCR to create a 1.2 kb DNA fragment, then digested with *Bam*HI, and run on an agarose gel. The presence of an insert fragment was confirmed by a Southern hybridization (Fig. 2, lane 4). This result showed that the recombinant plasmid, pHLA-17, was contained in the 6.43 kb *Bam*HI DNA fragment, including the *gD* gene. Watson *et al.* (1982) analyzed the 3.0 kb *Sac*I DNA fragment of the HSV-1 strain that was copied with the mRNA produced gD. Sisk *et al.* (1984) used the *gD* gene that was located in the *Eco*RI fragment of the HSV-1 strain Patton. This result indicated that the three groups used different DNA fragments and different strains for the *gD* gene source.

The amplified PCR products (1.2 kb DNA fragment), containing the *Bgl*II and *Eco*RI sites at the ends, were first cloned into the transfer vector pBacPAK9 in order to generate the pBac-gD plasmid. It was then transferred into the *Bgl*II and *Bam*HI sites downstream of the gX signal sequence of the pHcgXIIIB vector (Lee *et al.*, 1998) to generate the pHcGD recombinant plasmid (7.94 kb) (Fig. 1A). The insertion of the *gD* gene was also confirmed by the partial sequence of the *gD* gene-cloning site in the pHcGD plasmid, which identified a ligated *Bam*HI/*Bgl*II site (Fig. 1B). This finding indicated that the 1.2 kb DNA fragment was inserted in the correct orientation downstream of the gX signal sequence. The insertion of the *gD* gene fragments in the pHcGD was also confirmed by the digestion of the *Bam*HI and *Pvu*II enzymes and then a Southern blot analysis (Fig. 2, lane 6). These results indicated that the clone was positive for the *gD* gene

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ATGGGGGGGGCTGCCGCCAGGTTGGGGGCCGTGATTTTGTTTGTCGTCATAGTCGGCCTC   60
 M  G  G  A  A  A  R  L  G  A  V  I  L  F  V  V  I  V  G  L    20
 signal sequence (1-23aa)
CATGGGGTCCGCGGCAAATATGCCTTGGCGGATGCCTCTCTCAAGCTGGCCGACCCCAAT  120
 H  G  V  R  G  K  Y  A  L  A  D  A  S  L  K  L  A  D  P  N    40

CGCTTTCGCCGCAAAGACCTTCCGGTCCTGGACCAGCTGACCGACCCTCCGGGGGTCCGG  180
 R  F  R  R  K  D  L  P  V  L  D  Q  L  T  D  P  P  G  V  R    60

CGCGTGTACCACATCCAGGCGGGCCTACCGGACCCGTTCCAGCCCCCCAGCCTCCGATC  240
 R  V  Y  H  I  Q  A  G  L  P  D  P  F  Q  P  P  S  L  P  I    80

ACGGTTTACTACGCCGTGTTGGAGCGCGCCTGCCGCAGCGTGCTCCTAAACGCACCGTCG  300
 T  V  Y  Y  A  V  L  E  R  A  C  R  S  V  L  L  N  A  P  S   100

GAGGCCCCCCAGATTGTCCGCGGGGCCTCCGAAGACGTCCGGAAACAACCCTACAACCTG  360
 E  A  P  Q  I  V  R  G  A  S  E  D  V  R  K  Q  P  Y  N  L   120
                                                          *
ACCATCGCTTGGTTTCGGATGGGAGGCAACTGTGCTATCCCCATCACGGTCATGGAGTAC  420
 T  I  A  W  F  R  M  G  G  N  C  A  I  P  I  T  V  M  E  Y   140

ACCGAATGCTCCTACAACAAGTCTCTGGGGGCCTGTCCCATCCGAACGCAGCCCCGCTGG  480
 T  E  C  S  Y  N  K  S  L  G  A  C  P  I  R  T  Q  P  R  W   160
                *
AACTACTATGACAGCTTCAGCGCCGTCAGCGAGGATAACCTGGGGTTCCTGATGCACGCC  540
 N  Y  Y  D  S  F  S  A  V  S  E  D  N  L  G  F  L  M  H  A   180

CCCGCGTTTGAGACCGCCGGCACGTACCTGCGGCTCGTGAAGATAAACGACTGGACGGAG  600
 P  A  F  E  T  A  G  T  Y  L  R  L  V  K  I  N  D  W  T  E   200

ATTACACAGTTTATCCTGGAGCACCGAGCCAAGGGCTCCTGTAAGTACGCCCTCCCGCTG  660
 I  T  Q  F  I  L  E  H  R  A  K  G  S  C  K  Y  A  L  P  L   220

CGCATCCCCCCCGTCAGCCTGCCTGTCCCCCCAGGCCTACCAGCAGGGGGTGACGGTGGAC  720
 R  I  P  P  S  A  C  L  S  P  Q  A  Y  Q  Q  G  V  T  V  D   240

AGCATCGGGATGCTGCCCCGCTTCATCCCCGAGAACCAGCGCACCGTCGCCGTATACAGC  780
 S  I  G  M  L  P  R  F  I  P  E  N  Q  R  T  V  A  V  Y  S   260

TTGAAGATCGCCGGGTGGCACGGGCCCAAGGCCCCATACACGAGCACCCTGCTGCCCCCG  840
 L  K  I  A  G  W  H  G  P  K  A  P  Y  T  S  T  L  L  P  P   280

GAGCTGTCCGAGACCCCCAACGCCACGCAGCCAGAACTCGCCCCGGAAGCCCCCGAGGAT  900
 E  L  S  E  T  P  N  A  T  Q  P  E  L  A  P  E  A  P  E  D   300
                      *
TCGGCCCTCTTGGAGGACCCCGTGGGGACGGTGGCGCCGCAAATCCCACCAAACTGGCAC  960
 S  A  L  L  E  D  P  V  G  T  V  A  P  Q  I  P  P  N  W  H   320

ATACCGTCGATCCAGGACGCCGCGACGCCTTACCATCCCCCGGCCACCCCGAACAACATG 1020
 I  P  S  I  Q  D  A  A  T  P  Y  H  P  P  A  T  P  N  N  M   340

GGCCTGATCGCCGGCGCCGGTGGGCGGCAGTCTCCTGGCAGCCCTGGTCATTTGCGGAATT 1080
 G  L  I  A  G  A  V  G  G  S  L  L  A  A  L  V  I  C  G  I   360
 Transmembrane sequence (340-371aa)
GTGTACTGGATGCGCCGCCGCACTCAAAAAGCCCCAAAGCGCATACGCCTCCCCCACATC 1140
 V  Y  W  M  R  R  R  T  Q  K  A  P  K  R  I  R  L  P  H  I   380

CGGGAAGACGACCAGCCGTCCTCGCACCAGCCCCTGTTTTACTAGATACCCCCCCTTAAT 1185
 R  E  D  D  Q  P  S  S  H  Q  P  L  F  Y                     394
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Fig. 3. Nucleotide sequence of ORF of the HSV-1(F) *gD* gene (GenBank accession No. AF293614). The sequence of the coding strand is given. The predicted amino acid sequence was deduced from the base sequences. The *gD* gene in the pB-gD plasmid was fragmented with restriction enzymes, subcloned, then sequenced using the procedure of dideoxy chain termination. The translation initiation codon (ATG) and termination codon (TAG) are underlined. Hydropathy profile was determined using the values of Kyte and Doolite (1982). The potential hydrophobic signal and transmembrane sequences are underlined. The potential *N*-glycosylation sites are underlined and marked with *.

fragment.

The strategy for constructing a recombinant baculovirus shown by Lee *et al.* (1998a, 1998b) was used for the recombination of the gD gene into the HcNPV expression vector. The pHcGD plasmid, which contained the *gD* gene and linearized *lac*Z-HcNPV DNAs digested with the *Bsu*361 enzyme, were cotransfected into the *S. frugiperda* cells, according to the protocol described in Materials and Methods. The recombinant viruses were isolated using a plaque assay containing X-Gluc. No polyhedral inclusion bodies were formed in the infected cells. A white-colored plaque means no insertion of the *gD* gene, and a blue-colored plaque indicates a recombinant virus (Sambrook *et al.*, 1989). Four blue plaque clones, which were positive for the recombinant viruses, were isolated and characterized. Individual clones were named GD-HcNPV-1 to 4. The size of the plaques ranged from 2.0 mm to 4 mm in diameter (data not shown). The GD-HcNPV-1 clone, which exhibited the largest plaque size (4 mm), was used for further studies. These plaques were purified twice. The presence of the HSV-1 *gD* gene DNA in the recombinant baculovirus was confirmed by PCR (O'Reilly *et al.*, 1994) and a Southern blot hybridization. A 0.77 kb PCR product was obtained from the GD-HcNPV-1 recombinant virus and confirmed by a Southern blot analysis (Fig. 4). These results showed that the recombinant viruses contained the gD-encoding sequence and also that the *gD* gene was inserted correctly. The recombinant viruses multiplied normally in the *S. frugiperda* cells and produced the recombinant progeny (10^7 pfu per ml).

Sequence analyses of the *gD* gene The *gD* coding sequence, and deduced amino acid sequence, are shown in Fig. 3. The entire *gD* gene sequence was determined for both DNA strands. The open reading frame (ORF) of the HSV-1 strain F *gD* gene was 1.185 bp, and coded 394 amino acid residues. The translation start codon was ATG, and the termination codon was TAG. These results were consistent with the reports on the HSV-1 strain (Watson *et al.*, 1982). The *gD* gene sequence was highly conserved between the strains of HSV-1. The differences of *gD* gene sequences in the reported HSV-1 strain, and the differences within the coding region, are shown in Table 1. When compared with the previously reported HSV-1 *gD* gene sequence by Watson *et al.* (1982), five nucleotide differences in the HSV-1 strain F were found, of which four codons were changed in the coding region. This result indicated that the *gD* gene sequence of the strain F was highly conserved when compared with the known strain; also the differences may cause one of the specific characteristics between the strains.

The hydrophobicity profile of the gD polypeptide sequence, predicted according to Kyte and Doolites method (1982), was determined and shown in Fig. 3. The profile revealed the presence of two main hydrophobic regions. The first domain spanned from 1 to 23 amino acid residues that included a signal peptidase cleavage site (underlined in Fig. 3), which was predicted using the rules of Perlman and Halvorson (1983). The second hydrophobic domain was a region of 31 amino acid residues from position 340 to 371 (Fig. 3). The location of this sequence near the carboxy terminus of the gD protein may serve as a membrane anchor sequence (Watson *et al.*, 1982), and there are two polar residues, serine and threonine. The ORF was rich in proline (43 out of a total of 394 amino acids) and alanine (41 out of a total of 394 amino acids) residues. There were three *N*-glycosylation sites along the gD sequence (Fig. 3). The putative *N*-glycosylation sites (Asp-118, Asp-146, Asp-287) were underlined with an asterisk in Fig. 3. The glycosylated amino acids were distributed in the center of the polypeptide. The presence of a putative signal sequence, and transmembrane sequence with the *N*-glycosylation sites identified on the protein sequence, strongly suggests that the ORF codes for a glycoprotein.

Production of the fusion gX- gD proteins in insect cells The insect cells infected with the recombinant viruses GD-HcNPV-1 produced the gX-gD fusion proteins, which were confirmed by a radioimmunoprecipitation analysis (Fig. 5 lanes 2 and 3). Lane 2 is the immunoprecipitates of the lysate of cells infected with the recombinant viruses, and lane 3 is the immunoprecipitates of the concentrated cultured media infected with the recombinant viruses. Two lanes were densely immunoprecipitated with the gD- specific monoclonal antibody. It demonstrated that the gX-gD fusion protein was produced in the insect cells infected with the recombinant viruses and secreted into the culture media with approximately 54 kDa. However, the control groups were not immunoprecipitated with the specific monoclonal antibody (data not shown). The Pseudorabies virus gX glycoprotein accumulated in the medium of the infected cells has another

Table 1. Comparison of nucleotide and amino acid changes in the gD gene and protein from a different HSV-1 strain

Virus Strains	NT/amino acid changes at the following codons				
	4	36	44	365	369
F	GCT/A	CTG/L	CGC/R	CGC/R	CAA/Q
HSV-1*	ACT/T	ATG/L	GGC/G	CAC/H	CGG/R

The nucleotide (NT) sequence of the *gD* gene of HSV-1* was derived from the report of Watson *et al.*, (1982). Comparisons were made to the strain F nucleotide sequence and encoded amino acid sequence. Base differences are underlined. Codons are numbered from the initiation codon. A, Alanine; T, Threonine; L, Leucine; R, Arginine; G, Glycine; H, Histidine; Q, Glutamine.

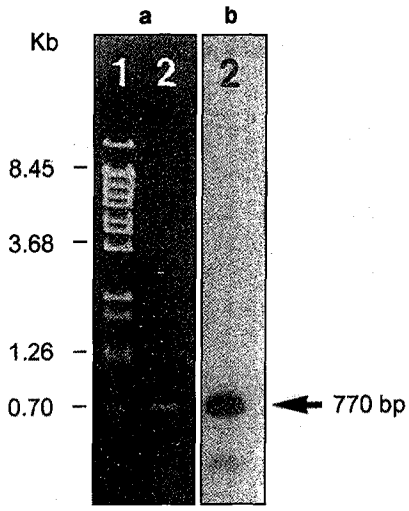

Fig. 4. PCR-based confirmation of the *gD* gene insertion in the GD-HcNPV-1 recombinant. Lanes a1, λ phage DNAs digested with the *Bst*EII; a2, PCR product of the GD-HcNPV-1 recombinant; and b2, Southern blot of the a2 DNAs. The PCR product (770 base pairs) were identified by a Southern blot analysis.

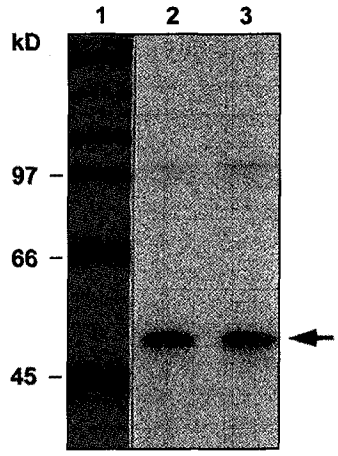

Fig. 5. Immunoprecipitations of the gD proteins produced from the virus-infected cell lysates and cultured media. Cells were infected with GD-HcNPV-1 viruses (m.o.i. = 2) for 4 days, labeled with L-^{3}H-leucine for 1 h and the cultured cells were centrifuged to separate the infected cells from the cultured media. The cell pellets were lysed by freeze-thawing. The cell lysates and the cultured media were dialyzed to 1.0 ml. The soluble fractions of the lysates and the media were mixed with Mab gD, respectively, then the antigen-antibody complexes were precipitated. The immunoprecipitates were resolved and run on 10% SDS-PAGE, and the labeled protein was detected by PPO fluorography. Lanes 1, standard molecular weight markers [myosin (200 kDa), β-galactosidase (116.2 kDa), phospholylase B (97.4 kDa), and bovine serum albumin (68 kDa)]; 2, immunoprecipitations of the cell lysates infected with the GD-HcNPV; and 3, immunoprecipitations of the cultured media infected with the virus. The right-hand arrow indicates the immunoprecipitates of the gD protein.

20-peptide sequence (2 kDa) at the N-terminus. This gX signal sequence might play an important role in the accumulation of the gX in the medium (Rea *et al.*, 1985). Our results also indicated that the gX signal sequence may play a role in the secretion of the gX-gD fusion protein from the insect cells infected with the GD-HcNPV-1 contained in the gX signal sequence. HSV-1 gD proteins were synthesized in *E. coli* cells with a molecular weight of approximately 46 kDa (Watson *et al.*, 1982). The gD protein, which were produced in the insect cells infected with *Autographa californica* nuclear polyhedrosis virus (AcNPV) recombinants containing the HSV-1 strain SC16 *gD* gene, was approximately 48 kDa (Krishna *et al.*, 1989). Sisk *et al.* (1994) also purified the gD protein synthesized in insect cells by baculovirus AcNPV recombinants containing the HSV-1 strain Patton *gD* gene and honeybee melittin signal sequence. The melittin signal peptide induced the synthesis of significant amounts of the gD intracellularly. This gD protein lacked carbohydrate and failed to fold properly. HSV-1 glycoproteins are present in infected cells in both a precursor and product form (Hogness and Roizman, 1975; Cohen *et al.*, 1978; Spear *et al.*, 1985). In this result, the molecular weight of the gD was 54 kDa; however, the proteins isolated by the later two groups was about 48 kDa. The differences may be caused by the gX signal sequence (2 kDa) in the HcNPV vector system, as well as a glycosylation and/or other modifications during the maturation of the precursor polypeptide. Our results indicated that the recombinant virus GD-HcNPV-1 expressed the glycosylated mature gD in the insect cells, and was then secreted into the culture media.

The production of the gX-gD fusion protein in the *S.*

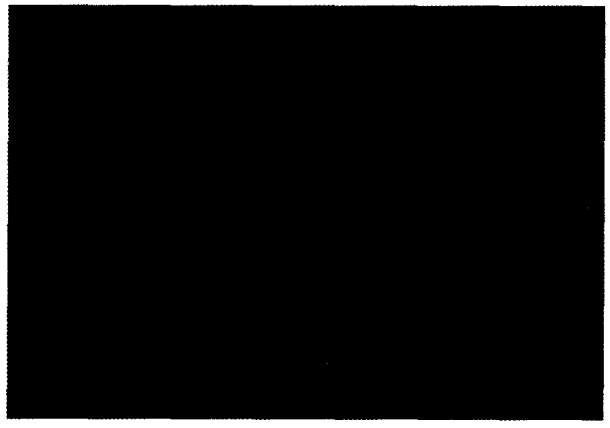

Fig. 6. Immunofluorescence of the gD fusion protein produced in *S. frugiperda* cells infected with the GD-HcNPV-1 recombinant virus. The virus-infected cells were fixed in acetone and treated with an anti-gD monoclonal antibody followed by a fluorescein isothiocyanate-conjugated sheep anti-mouse antibody IgG. The fluorescent cells were observed with a fluorescent microscope (×200). The arrowed cells exhibit high surface fluorescence with a green color.

frugiperda cells that were infected with the GD1-HcNPV-1 was also detected by an indirect immunofluorescence analysis using a fluorescein-conjugated gD monoclonal antibody. No fluorescence was seen in the cells infected with the wild-type baculovirus *lac*Z-HcNPV (data not shown); however, the GD-HcNPV-1 infected cells exhibited a high surface fluorescence with a green color (Fig. 6). The green fluorescence indicated that the gD protein was transported at the cell surface, which is consistent with the report of Ghiasi *et al.* (1991) and Kang *et al.* (2000).

In conclusion, these results demonstrate that the HSV-1 strain F *gD* gene was localized in the 6.43 kb *Bam*HI fragment of the HSV-1 (F) genome. Also, GD-HcNPV recombinant viruses produce a mature gX-gD fusion protein by the gene transfer in insect cells, and the fusion proteins were secreted into the medium, which indicates the utility of the HcNPV-insect cell system for producing and characterizing eukaryotic proteins. The ORF of the fused gX-*gD* gene encoded for a polypeptide of 54 kDa that had several characteristics of a glycoprotein.

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References

Adams, G. A. and Rose, J. K. (1985b) Structural requirements of a membrane-spanning domain for protein anchoring and cell surface transport. *Cell* **41**, 1007-1015.

Baines, J. D. and Roizman, B. (1993) The U_L10 gene of *Herpes simplex* virus 1 encodes a novel viral glycoprotein, gM, which is present in the virion and in the plasma membrane of infected cells. *J. Virol.* **67**, 1441-1452.

Birnboim, H. C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**, 1513-1523.

Blacklaws, B. A., Nashi, A. A. and Darby, G. (1987) Specificity of the immune response of mice to *Herpes simplex* virus glycoproteins B and D constitutively expressed on L cell lines. *J. Gen. Virol.* **68**, 1103-1114.

Bollag, D. M., Rozycki, M. D. and Edelstein, S. J. (1996) *Protein Methods*, 2nd ed., pp. 96-130, Wiley-Liss, New York.

Cha, S. C., Kang, H., Lee, S.Y., Park, K. J. and Lee, H. H. (2000) Expression of HSV-1 (F) glycoprotein B gene in insect cells infected by HcNPV recombinant. *J. Microbiol. Biotechnol.* **10**, 355-362.

Cho, G., Na, S., Suh, S. W. and Jung, G. (2000) Expression of recombinant HBV pol proteins in HepG2 cells. *J. Biochem. Mol. Biol.* **33**, 440-447.

Cohen, G. H., Katze, M., Hydren-Stern, C. and Eisenberg, R. J. (1978) Type-common C11 antigen of *Herpes simplex* virus is associated with a 59,000-molecular-weight envelope glycoprotein. *J. Virol.* **27**, 172-181.

Chung, H. K., Lee, S. H., Kim, S. Y. and Lee, H. H. (1994) Nucleotide sequence analysis of the RNA-dependent RNA polymerase gene of infectious pancreatic necrosis virus DRT strain. *J. Microbiol. Biotechnol.* **4**, 264-269.

Eisenberg, R. J., Manuel, D. L., DE Leon, P., Matthews, J. T., Spear, P. G., Gibson, M. G., Lasky, L. A., Berman, P., Golub, E. and Cohen, G. H. (1985) Localization of epitopes of *Herpes simplex* virus type 1 glycoprotein D. *J. Virol.* **53**, 634-644.

Ejercito, P. M., Kieff, E. D. and Roizman, B. (1968) Characterization of *Herpes simplex* strains differing in their effects on social behavior of infected cell. *J. Gen. Virol.* **2**, 357-364.

Fuller, A. O. and Spear, P. G. (1985) Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of *Herpes simplex* virus to cells and lack of inhibition by potent neutralizing antibodies. *J. Virol.* **55**, 475-482.

Highlander, S. L., Sutherland, S. L., Gage, P. J., Johnson, D. C., Levine, M. and Gloriosol, L. C. (1987) Neutralizing monoclonal antibodies specific for *Herpes simplex* virus glycoprotein D inhibit virus penetration. *J. Virol.* **61**, 3356-3364.

Ghiasi, H., Nesburn, A. B. and Wechsler, S. L. (1991) Cell surface expression of *Herpes simplex* virus type 1 glycoprotein H in recombinant baculovirus-infected cells. *Virology* **185**, 187-194.

Grunstein, M. and Hogness, D. (1975) Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. USA* **72**, 3961-3965.

Hogness, R. W. and Roizman, B. (1975) Proteins specified by *Herpes simplex* virus. XIII. Glycosylation of viral polypeptides. *J. Virol.* **16**, 1308-1326.

Johnson. D. C. and Ligas, M. W. (1988) *Herpes simplex* Viruses lacking glycoprotein D are unable to inhibit virus penetration: Quantitative evidence for virus-specific cell surface receptors. *J. Virol.* **62**, 4605-4612.

Kang, H., Cha, S. C., Han, Y. J., Park, I. H., Lee, M. J., Byun, S. M. and Lee, H. H. (2000) High level production of glycoprotein H of HSV-1(F) using HcNPV vector system. *J. Biochem. Mol. Biol.* **33**, 483-492.

Kang, H., Park, K. J., Cha, S. C., Kim, S. Y., Yang, K. S., Kim, N. J. and Lee, H. H. (1996) Cloning of thymidine kinase gene of *Herpes simplex* virus type-1. *J. Kor. Soc. Virol.* **26**, 121-129.

Kornfeld, R. and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* **54**, 631-664.

Krishna, S., Blacklaws, B. A., Overton, H. A., Bishop, D. L. and Nash, A. A. (1989) Expression of glycoprotein D of *Herpes simplex* virus type 1 in a recombinant baculovirus: Protective responses and T cell recognition of the recombinant-infected cell extracts. *J. Gen. Virol.* **70**, 1805-1814.

Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132.

Lee, H. H. (1987) Replication and cloning of *Hyphantria cunea* nuclear polyhedrosis virus in *Spodoptera frugiperda* cell line. *Hanguk J. Genetic Engineering* **2**, 1-6.

Lee, H. H., Kang, B. J. and Park, K. J. (1998a). Construction of a Baculovirus expression system using *H. cunea* nuclear polyhedrosis virus for eukaryotic cell. *J. Microbiol. Biotechnol.* **8**, 676-684.

Lee, H. H., Kang, H., Kim, J. W. and Song, J. Y. (1999) Construction of a transfer baculovirus vector containing gX signal sequence of Pseudorabies virus and a recombinant. *J. Microbiol. Biotechnol.* **9**, 541-547.

Lee, D. and Kim, D. H. (1999) Heterologous expression of lignin peroxidase H2 in *Escherichia coli*: In vitro refolding and activation. *J. Biochem. Mol. Biol.* **32**, 486-491.

Lee, H. H. and Lee, K. W. (1988) Isolation, complementation and partial characterization of temperature-sensitive mutants of the Baculovirus *Hyphantria cunea* nuclear polyhedrosis virus. *J. Gen. Virol.* **69**, 1299-1306.

Lee, H. H. and Miller, L. K. (1978) Isolation of genotypic variants of *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* **27**, 754-767.

Lee, H. H., Min, B. H., Chung, H. K., Lee, K. K., Park, J. K., Cha, S. C. and Seo, N. S. (1992) Genomic structure and nucleotide sequence of polyhedrin gene of *Hyphantria cunea* nuclear polyhedrosis virus genome DNA. *Mol. Cells* **2**, 303-308.

Lee, H. H., Moon, E. S., Lee, S. T., Hwang, S. H., Cha, S. C. and Yoo, K. H. (1998b) Construction of a Baculovirus *Hyphantria cunea* NPV insecticide containing the insecticidal protein gene of *Bacillus thuringiensis* subsp. *kurstaki* HD1. *J. Microbiol. Biotechnol.* **8**, 685-691.

Mandel, M. and Higa, A. (1970) Calcium dependent bacteriophage DNA phage infection. *J. Mol. Biol.* **53**, 154-162.

Martin, S. and Rouse, B. T. (1987) The mechanism of antiviral immunity induced by a vaccinia virus recombinant expressing *Herpes simplex* virus type 1 glycoprotein D: Clearance of local infection. *J. Immunol.* **138**, 3431-3440.

O'Reilly, D. R., Miller, L. K. and Luckow, V. A. (1994) *Baculovirus expression vectors, A laboratory manual.* Oxford University Press, New York and Oxford.

Perlman, D. and Halvorson, H. O. (1983) A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptidase. *J. Mol. Biol.* **167**, 391-409.

Rea, T., Timmis, J. G., Long, G. W. and Post, L. E. (1985) Mapping and sequence of the gene for the Pseudorabies virus glycoprotein which accumulates in the medium of infected cell. *J. Virol.* **54**, 21-29.

Sambrook, L., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning; A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci., USA* **74**, 5463-5467.

Sisk, W. P., Bradley, J. D., Leipold, R. J., Stoltzfus, A. M., DE Leon, M. P., Hilf, M., Peng, C., Cohen, G. H. and Eisenberg, R. J. (1994) High-level expression and purification of secreted forms of *Herpes simplex* virus type 1 glycoprotein gD synthesized by baculovirus-infected insect cells. *J. Virol.* **68**, 766-775.

Smith, G. E., Fraser, M. J. and Summers, M. D. (1983) Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: Deletion mutations within the polyhedrin gene. *J. Virol.* **46**, 584-593.

Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **69**, 503-517.

Spear, P. G. (1985) Glycoproteins specified by herpes simplex viruses. In B. Roizman, ed., *The Herpesviruses,* Vol. 3. pp. 315-356, New York and London, Plenum Press.

Wachsman, M., Luo, J. H., Aurelian, L., Perkus, M. E. and Paoletti, E. (1989) Antigen-presenting capacity of epidermal cells infected with vaccinia virus recombinants containing the *Herpes simplex* virus glycoprotein D, and protective immunity. *J. Gen. Virol.* **70**, 2513-2520.

Watson, R. J., Weis, J. H., Salstrom, J. S. and Enquist, L. W. (1982) *Herpes simplex* virus type 1 glycoprotein D gene: nucleotide sequence and expression in *E. coli. Science* **218**, 381-383.