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High Level Production of Glycoprotein H of HSV-1 (F) Using HcNPV Vector System

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The *Herpes simplex* virus type 1 (HSV-1) strain F glycoprotein H (*gH*) gene in the pHLB-4 plasmid was recombinated into a baculovirus expression vector (*lacZ*-HcNPV) to construct a recombinant virus GH-HcNPV expressing gH. The sequences of gH and its expression were analyzed. The *gH* gene was located in the 6.41 kb *Bgl*II fragment. The open reading frame (ORF) of the *gH* gene was 2,517 bp and codes 838 amino acid residues. Insect cells infected with this recombinant virus synthesized a high level of the matured and gX-gH fusion protein with approximately 112 kDa. The fusion gH protein was localized on the membrane of the insect cells as seen by using immunofluorescence assay and accumulated in the cultured media by the SDS-PAGE and immunoprecipitation assays. The 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. Antibodies raised in mice to this recombinant protein recognized viral gH and neutralized the infectivity of HSV-1 *in vitro*. These results demonstrate that it is possible to produce a mature protein by gene transfer in eukaryotic cells, and indicate the utility of the HcNPV-insect cell system for producing and characterizing eukaryotic proteins. Furthermore, the neutralizing antibodies would appear to protect mice against HSV; accordingly, this particular recombinant protein may be useful in the development of a subunit vaccine.

Keywords: Baculovirus, Glycoproteins, Glycosylation, HSV-1, Neutralizing antibody.

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Introduction

Herpes simplex virus type 1 (HSV-1) produces at least eleven glycoproteins embedded in the virion envelopes and cell membranes of infected cells. They are known antigenically and functionally to be distinct species and are designated as gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM (Spear, 1976; Para *et al.*, 1982; Ackerman *et al.*, 1986; Gompels and Minson, 1986; Longnecker *et al.*, 1987; Ramsawamy and Holland, 1992; Bains and Roizman, 1993). The HSV-1 glycoproteins appear to be the primary inducers and targets of humoral and cell-mediated immune responses to HSV-1 infection (Norrild, 1985; Spear, 1985). Only two glycoproteins, gB and gH of the *Herpes simplex* virus, are found in herpesviruses of all subgroups (Bzik *et al.*, 1984; Gompels and Minson, 1986). In HSV-1 infected cells, gH plays an important role in viral egress from the cells, entry into cell, and membrane fusion (Buckmaster *et al.*, 1984b; Gompels and Minson, 1986; Richman *et al.*, 1986; Fuller *et al.*, 1989). In tissue culture, the antibody to gH can neutralize virus infectivity, prevent cell to cell spread of the virus, and block fusion of the virion envelope with the plasma membrane (Gompels and Minson, 1986; Fuller *et al.*, 1989; Blacklaws *et al.*, 1990). HSV-1 gH is a transmembrane protein of approximately 110 kDa, and located on the virion envelope and on the surface of infected cells (Buckmaster *et al.*, 1984b; Gompels and Minson, 1986; Desai *et al.*, 1988).

The nucleotide sequences of the *gH* gene of the HSV-1 strain HFEM and SC10 (Gompels and Minson, 1986), strain 17 (McGeoch and Davison, 1986) and strain 103/65 (Buckmaster *et al.*, 1984a) were determined and analyzed. Based on the DNA sequence analysis, the *gH* gene encodes a polypeptide of 838 amino acids with an estimated molecular weight of 110 kDa (Showalter *et al.*, 1981; Buckmaster *et al.*, 1984a; Gompels and Minson, 1986; Desai *et al.*, 1988).

The gH is an important target for both humoral and cell-mediated immune responses to HSV-1 infection (Gompels

and Minson, 1986; Ghiasi *et al.*, 1994). Prophylactic immunization of naive animals with gH has provided protection against primary infection and reduced latency (Chan, 1983; Gompels *et al.*, 1991). Therefore gH is one of the principal candidates for a HSV subunit vaccine. Gompels *et al.*, (1989) characterized gH using a mammalian cell expression system. Baculovirus expression vectors have proven useful for achieving high-level expression of a variety of foreign genes in insect cells (Smith *et al.*, 1983; Summers and Smith, 1988; Ghiasi and Nesburn, 1991; O'Reilly and Miller, 1994). The *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) (Lee, 1987; Lee and Lee, 1988) is one of the baculovirus group. We reported construction of baculovirus transfer and expression vectors based on HcNPV in order to achieve a high level of expression of a variety of foreign genes in *Spodoptera frugiperda* cells (Lee *et al.*, 1998ab, 1999). Specifically, our current study was designed to study the high level expression, sequence analysis, and properties of gH of a moderate pathogenic HSV-1 strain F, as compared to others (Ejercito *et al.*, 1968) as a subunit vaccine. Accordingly, using these systems, gH coding sequence of HSV-1 (F) was recombined into the expression vector and then the resulting gH protein was characterized. Further more, it was then demonstrated that immunization of mice with this recombinant gH induces immune response.

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 and HSV-1 Kos was obtained from the Korean National Institute of Health, Seoul, Korea. Vero cell (ATCC CCL81) was obtained from the Korean Type Collection (KTCC). The HSV-1 strains were grown at 37°C in the Vero cells using a Eagle's minimum essential medium (Gibco, Detroit, USA) with 10% fetal bovine serum (Gibco) as previously described by Kang *et al.*, (1996). The *lac*Z-*Hyphantria cunea* nuclear polyhedrosis virus (*lac*Z-HcNPV) containing a deletion in polyhedrin gene (Lee *et al.*, 1998a) in this laboratory was propagated in the *Spodoptera frugiperda* cells (IPLB-SF-21) obtained from L. K. Miller, University of Georgia, USA, were propagated 27°C in a TC-100 medium (Gibco, Middletton, USA) as previously described by Lee *et al.*, (1998ab). The cells were routinely maintained in a TC-100 medium (Gibco) supplemented with 0.2% tryptose broth powder and 10% fetal bovine serum and passaged every 5 days. The virus was titered for infectivity as described by Lee *et al.*, (1987). The virus inoculum used was extracellular nonoccluded virus (NOV) derived from a cell culture medium (Lee and Miller, 1978).

Bacterial strains and plasmids The pHcgXIIIB plasmid harboring in the *E. coli* XL1-Blue (Lee *et al.*, 1999) was used for HSV-1 gH gene cloning and transferring into expression viral vector (*lac*Z-HcNPV) (Lee *et al.*, 1998a). The pBluescript SK (+) plasmid (Stratagene, La Jolla, USA) was used for cloning or sequencing. The pHLB-4 plasmid bearing in *E. coli* XL1-Blue (Kang *et al.*, 1996) was used for a HSV-1 gH gene source and was maintained in LB broth (Gibco, Gaithersburg, USA).

Oligonucleotides The oligonucleotide primers (H-1, 5':5'-GGGAGATCTATGGGGAATGGTTTATGGTTC-3' and H-2, 3':5'-GCCAGATCTTTTATTCGCGTCTCCAA-3'), (H-3: 3':5'-GGAGAAATACACGTA-3') and a probe (H-4, 5'-ACGGGGTCC CCCATGGGGAATGGTTTATGG-3') were deduced from terminal sequences of the published sequences of the gH gene of the HSV-1 strain HFEM (Gompels *et al.*, 1986) and used for the PCR and colony hybridization, respectively. The primer H-1 contained *Bgl*II site (underlined) and translation start codon (bolded), and the primer H-2 contained *Bgl*II site and coding termination codon UAA (bolded). The primers (Hc-1, 3:5'-TGTTAACCTTCTCCC-3' and Hc-2, 5:5'-CACGTCGAGTCAAT TG TAC-3') were deduced from a terminal sequence of the published sequence of polyhedrin gene of HcNPV (Lee *et al.*, 1992) and were used for the partial sequencing of the gene inserted in the HcNPV transfer vector. The oligonucleotides were synthesized by Korea Biotec Inc., Daejeon, Korea.

Monoclonal antibody The HSV-1 gH specific monoclonal antibody 52S was obtained from Dr. U. Gompels, University of London, UK, and fluorescein isothiocyanate-conjugated sheep anti-mouse IgG was purchased from Boeringer Mannheim Biochemicals (Indianapolis, USA).

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

Colony and southern blot hybridizations Colony hybridization for detection of colonies containing *gH* gene fragments of the HSV-1 *Bgl*II genome library (Kang *et al.*, 1996) was carried out according to the procedure described by Grunstein and Hogness (1975) using the probe H-4. The *gH* locus in the selected recombinant plasmids was confirmed by a Southern blot analysis (Southern, 1975).

Amplification of DNAs A polymerase chain reaction (PCR) was performed according to the procedure described by Chung *et al.* (1994). PCR was done with 1.0 mg of template DNA, 0.25 mM each primer, 200 mM deoxynucleoside triphosphates and 2 ml DMSO. The mixture solution was heated to 100 for 7 min on ice for 10 min and added 1 unit of Taq polymerase was added. Thirty cycles of amplification were performed and the last cycle was passed on a Perkin-Elmer Thermocycler. Aliquots of the amplification assays (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 DNA genome and vector DNAs were digested and electrophoresed on a 1.0% agarose gel. The molecular size of each DNA fragment was determined by comparing their mobility with a *Bst*EII or *Hin*dIII-

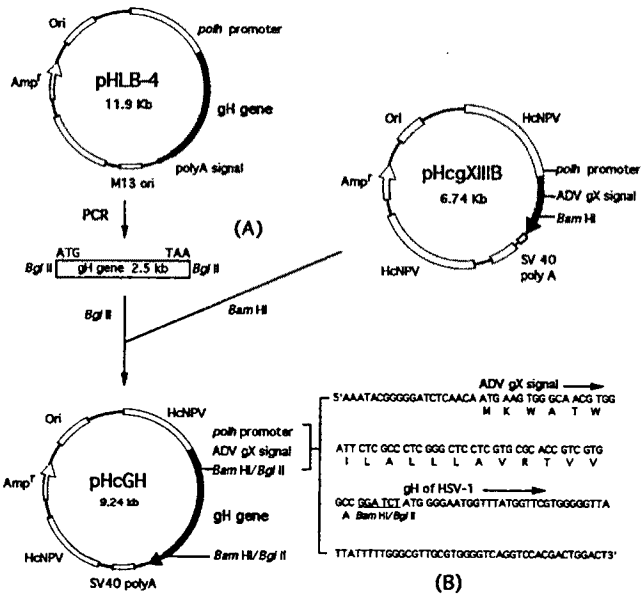

Fig. 1. Construction scheme for pHcGH plasmid containing gH gene sequence. (A) The 2.5 kb *gH* gene sequence in the plasmid pHLB-4 was amplified with PCR, which were cloned into pHcgXIIIB transfer vectors to construct the pHcGH recombinant plasmid. (B) Partial nucleotide sequence of the cloning site downstream of the polyhedrin promoter region. The part of the downstream of the promoter, the ligated *Bam*HI/*Bgl*II site, and the translation start codon ATG of the *gH* gene were apparent.

digested phage λ DNA fragments. The reactions were terminated by the addition of a 1/10 volume of the stop solution (Sambrook *et al.*, 1989). Details of the gel electrophoresis and visualization of the DNA fragments were previously described by Lee and Miller (1978). The DNA fragments were fractionated and purified in 1.0% low-melting- temperature-agarose gels containing Tris-borate and ethidium bromide (Lee *et al.*, 1998b, 1990).

Cloning and transformation The HSV-1 gH gene in the plasmid pHLB-4 (Kang *et al.*, 1996) was amplified by a PCR and a recombinant plasmid (pHcGH) was constructed using standard protocols (Sambrook *et al.*, 1989; Lee *et al.*, 1998a, 1998b) as detailed in Fig. 1. The plasmid pHLB-4 was amplified with two primers containing *Bgl*II sites at the ends to create a 2.5 kb of gH gene sequence. This gene was then inserted into the *Bam*HI site of the baculovirus transfer vector pHcgXIIIB. The resulting recombinant was named pHcGH plasmid (Fig. 1A). Competent *E. coli* cells were prepared and transformed by the procedure described by Mandel and Higa (1970).

Construction and selection of HcNPV 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 the linearized *lac*Z-HcNPV DNA with *Bsu361* enzyme and 10 μl (10 μg) of pHcGH plasmid DNA in TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH7.5) in 40 μl H_2O to construct recombinant viruses using lipofectin-transfection as described by Lee *et al.*, (1998ab). The recombinant viruses in the supernatants was selected by 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. Blue occlusion-negative plaques were picked and added to a serum-free medium. Insertion of the gH gene in the recombinant virus was analyzed by a PCR using primers Hc-1 and Hc-2 and a Southern blot (Southern, 1975).

Nucleotide sequencing The dideoxy chain termination sequencing (Sanger *et al.*, 1977) was performed on a polyacrylamide sequencing gel using approximately 8 μg/μl of double-stranded DNA templates and 100 pmol/μl of limiting primer. The DNA fragments were labeled with ^{35}S-ATP (Amersham-pharmacia, Buckingham Shire, UK) and sequenced with a 7-deaza-dGTP sequencing kit with a Sequenase version 2.0 T7 DNA polymerase (United State Biochemical, Cleaveland, USA) using pBluescript T3 primer, T7 DNA primer, and M13 reverse primer.

SDS-PAGE for detection of gH protein Proteins were fractionated on 7.5% polyacrylamide gels as described by Bollag *et al.* (1996) and Kang *et al.* (2000). *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 gH 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 were treated in the same manner as the infected cells as control. The samples were loaded for PAGE analysis.

Radioimmunoprecipitation assay for detection of gH protein A radioimmunoprecipitation assay was carried out using a modification of the procedures described by Gompels and Minson (1989), Cha *et al.* (2000) and Park *et al.* (2000). *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. Each cell was infected with the glycoprotein gene recombinant virus at a multiplicity 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 the TC-100 medium for 48 h at 27°C. After incubation, the monolayers were washed twice in 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 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 a immunoprecipitation buffer and incubated with antibody for the antigen and antibody complex formation. 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 proteins in cells was carried out using a modification of the procedures described by Gompels and Minson (1989), 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 recombinant baculoviruses expressing gH 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 gH-specific 52S 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 and then incubated for 1 h with a 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), and then examined through a fluorescent microscope (AO Fluoro- star 20, USA).

Preparation of cell lysates for immunization The *S. frugiperda* cells (1×10^6 cells per flask) were seeded in eight flasks (60 mm), allowed to attach in a monolayer overnight at 27°C, infected with the recombinant baculoviruses expressing gH at a multiplicity of infection of 1.0 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 postinfection were freeze-thawed, lyophilized and resuspended in 2 ml of a PBS buffer (pH 6.2) to a final concentration of 8×10^6 cells. The *S. frugiperda* cells infected with the wild-type *lac*Z-HcNPV were treated in the same manner as the control infected cells.

Immunization of mice The mice immunization was carried out using a modification of the procedures described by Ghiasi *et al.* (1991, 1992) and Kim *et al.* (1999). Ten mice (Balb/c strain, 6-8 weeks old) (Daehan laboratory animal research center, Chungnam, Korea) were vaccinated with three times at 3-week intervals both subcutaneously and intraperitoneally with freeze-thawed whole cell lysates of *S. frugiperda* cells infected with recombinant baculovirus expressing gH. 250 µl (10^6 cells) of the cell lysates was injected subcutaneously with Freund's complete adjuvant at 1 : 1 ration on Day 0 and an identical preparation with Freund's incomplete adjuvant on Days 21 and 42, and Intraperitoneal (ip) injections were given concurrently with 250 µl (10^6 cells) of the lysates in PBS. Ten mock-vaccinated mice were inoculated with the *S. frugiperda* cell lysates infected with the wild-type baculovirus using the same regimen. A positive control group of 10 mice was immunized three times ip with 2×10^5 pfu of the nonvirulent HSV-1 strain KOS. The sera were collected at 3 weeks after the final vaccination and pooled for each group.

Serum neutralization assay A virus neutralization assay was performed using a modification of the procedure described by Martin and Rouse (1987). Briefly, 100 µl of heat-inactivated serum was added in a serial dilution on a 96-well, flat-bottom microtiter plate (Falcon, Lincoln Park, USA). 100 µl of $200 \times TCID_{50}$ of the live HSV-1 strain F was added to the plate, sealed and incubated for 18 h at 4°C. Then, 100 µl of 5×10^3 viable vero cells were added to the plates, which were incubated at 37°C for 5 days. The serum dilution factor that neutralized 50% of the virus was determined as the titer. $TCID_{50}$ was 0.69 pfu as determined by the procedure described by Reed and Muench (1938).

Results and Discussion

Construction of a recombinant virus, GH1-HcNPV, expressing gH A *Bgl*II fragment library of the HSV-1 strain F DNA genome was screened with a H-4 probe. A number of positive colonies were identified and one colony, which strongly hybridized with this probe, was isolated. The purified DNA of the clone was digested with *Bgl*II and run on an agarose gel. The presence of an insert fragment was confirmed by Southern hybridization (Fig. 2). This result showed that the recombinant plasmid, pHLB-4 (Kang *et al.*, 1996), contained the 6.41 kb *Bgl*II DNA fragment harboring the gH gene. Also Gompels and Minson (1986) analyzed the 6.4 kb *Bgl*II "m" fragment of HSV-1 strain HFEM, and revealed the location of a gH gene with thymidine kinase and 57 kDa genes on the "m" fragment. We found that the *gH* genes of the HSV-1 strains F and HFEM were located in a similar size to the 6.4 kb *Bgl*II fragment. But, McGeoch and Davison (1986) found the *gH* gene located in *Eco*RI "g" clone and *Bam*HI "p" clone of HSV-1 strain 17.

For cloning of the HSV-1 *gH* gene coding for the 110 kDa polypeptide (Showalter *et al.*, 1981) into the baculovirus transfer vectors, the *gH* gene in the plasmid pHLB-4 was amplified by a PCR to generate a 2.5 kb fragment of *gH* gene sequence and then cloned into the baculovirus transfer vector

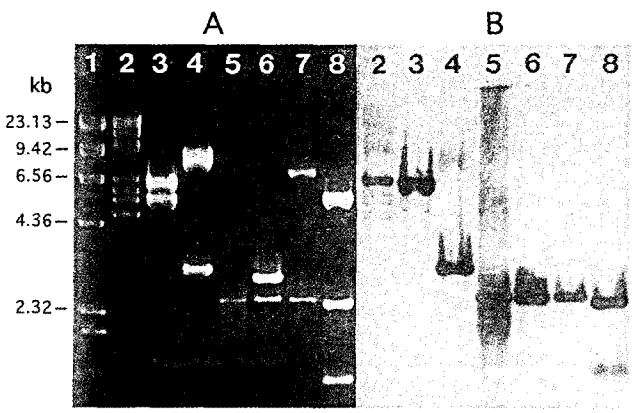

Fig. 2. Agarose gel analysis of *gH* gene fragments (A) and identifications of *gH* gene fragments in the recombinant clones by Southern blot analysis (B). The digested DNAs were electrophoresed for 17 h on a 1.0% agarose gel. Lanes 1, λ phage DNAs digested with *Hind*III; 2, HSV-1 genomic DNAs digested with *Bgl*II; 3, pHLB-4 plasmid DNAs digested with *Bgl*II; 4, pHLB-4 plasmid DNAs digested with *Apa*I; 5, PCR DNAs of *gH* gene; 6, pBlu-GH plasmid DNAs double-digested with *Hind*III plus *Xba*I; 7, pHcGH(+) plasmid DNAs with *Bgl*II. The probes used here was the *Asp*I 3.1 kb fragment containing *gH* gene sequence.

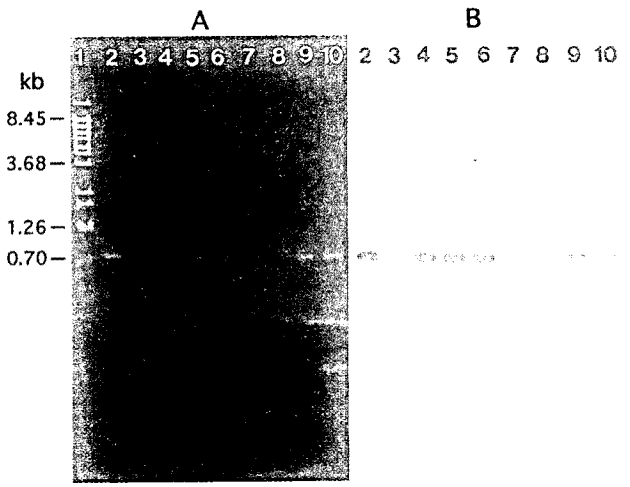

Fig. 3. PCR-based identification of the inserted *gH* gene of the GH1-HcNPV recombinants. The PCR products of each recombinant virus were electrophoresed on a 1.0% agarose gel for 2 h. The primers, Hc-1 and H-3, derived from HcNPV were used for the PCR identification of the inserted *gH* gene. The PCR products were about 770 bp. Lanes 1, λ phage DNAs digested with *Bst*EII; 2~10, GH1-HcNPV-1~9 viral clones, respectively. Panel B shows the results of Southern blots analysis of the panel A.

to generate the pHcGH recombinant plasmid (Fig. 1A). Partial sequence of the *gH* gene insertion site in the pHcGH was determined (Fig. 1B). Analysis of the cloning site, *Bam*HI/*Bgl*II, of the pHcGH clone showed that the 2.5 kb was inserted in the correct orientation downstream of the gX signal sequence of the Pseudorabies virus and fused with the gX signal sequence. Insertion of the *gH* gene fragments in the pHcGH was also confirmed by a Southern blot analysis (Fig. 2 lane 7). These results indicate that the each clone was positive for the *gH* gene fragment. Analysis of the pHcGH recombinant plasmid by eight restriction enzymes showed that the gH gene was inserted in the correct orientation downstream the polyhedrin promoter (data not shown). The 2.5 kb *gH* gene fragment was also mapped by digestion with restriction enzymes.

The strategy for the construction of the recombinant baculovirus described in Lee *et al.* (1998ab) was used for cloning of the *gH* gene. The pHcGH transfer vector, containing the gH gene and the linearized *lac*Z-HcNPV DNAs digested with the *Bsu*361 enzyme, were cotransfected into *S. frugiperda* cells according to the protocol described in Materials and Methods. The recombinant viruses were isolated by a plaque assay containing X-Gluc. No polyhedral inclusion bodies were formed in the infected cells. White-colored plaque means no insertion of the gH gene, and a blue-colored plaque indicates a recombinant virus (Sambrook *et al.*, 1989). Nine blue plaque clones, which were positive for recombinant viruses, were isolated and characterized. Individual clones were named GH1-HcNPV-1 to 9 on the basis of their selected order. The size of plaques ranged from 2.0 mm to 4 mm in diameter (data not shown). The GH1-HcNPV-1 clone that exhibited the largest plaque size (4 mm) was used for further studies. This plaque was plaque-purified twice. The presence of the HSV-1 *gH* gene DNA in the recombinant baculovirus was confirmed by a PCR (O'Reilly *et al.*, 1994) and Southern blot hybridization. From the six clones (clones 1, 3, 4, 5, 8 and 9) a 0.77 kb PCR product that was comprised of the partial sequence of the gH gene was obtained. Southern blot analysis showed this to be *gH* gene product (Fig. 3). These results showed that the recombinant viruses contained the gH encoding sequence and the *gH* gene was inserted correctly.

Table 1. Comparisons of gH gene nucleotide sequences from different HSV-1 strains

NT locos	NT difference in the strains				
	F	103/65	17	SC16	HFEM
44	T	C	A	A	C
84	C	T	-	-	T
112	A	G	G	G	G
355	C	-	-	A	-
412	T	-	-	-	G
439	T	C	C	C	C
448	G	-	-	A	A
503	A	G	-	-	-
546	G	-	-	-	A
612	C	-	-	-	T
851	C	T	T	-	-
1284	C	-	-	A	-
1596	G	-	T	-	-
1650	A	-	C	C	C
1717	G	-	-	A	-
1792	A	G	G	G	G
1881	C	-	T	T	-
2242	C	-	-	T	T
2301	C	G	A	A	A

NT: nucleotide. "-": the identical nucleotide with the F strain

Sequence analyses of the *gH* gene and protein of HSV-1 strain F The PCR product of the *gH* gene in the pHLB-4 clone was digested with *the Bgl*II enzyme and cloned into the *Bam*HI site of pBluescript SK (+) and named pBlu-GH (Fig. 2 lane 6). The *gH* gene in the pBlu-GH plasmid was digested with *Xho*II, *Sma*I, *Eco*RI and *Bst*XI 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 and the amino acid sequence was deduced. The entire *gH* sequence was determined for both strands of the DNA. An open reading frame (ORF) of the *gH* gene was 2,517 bp and codes 838 amino acid residues (Fig. 4). The translation start codon was ATG and termination codon was

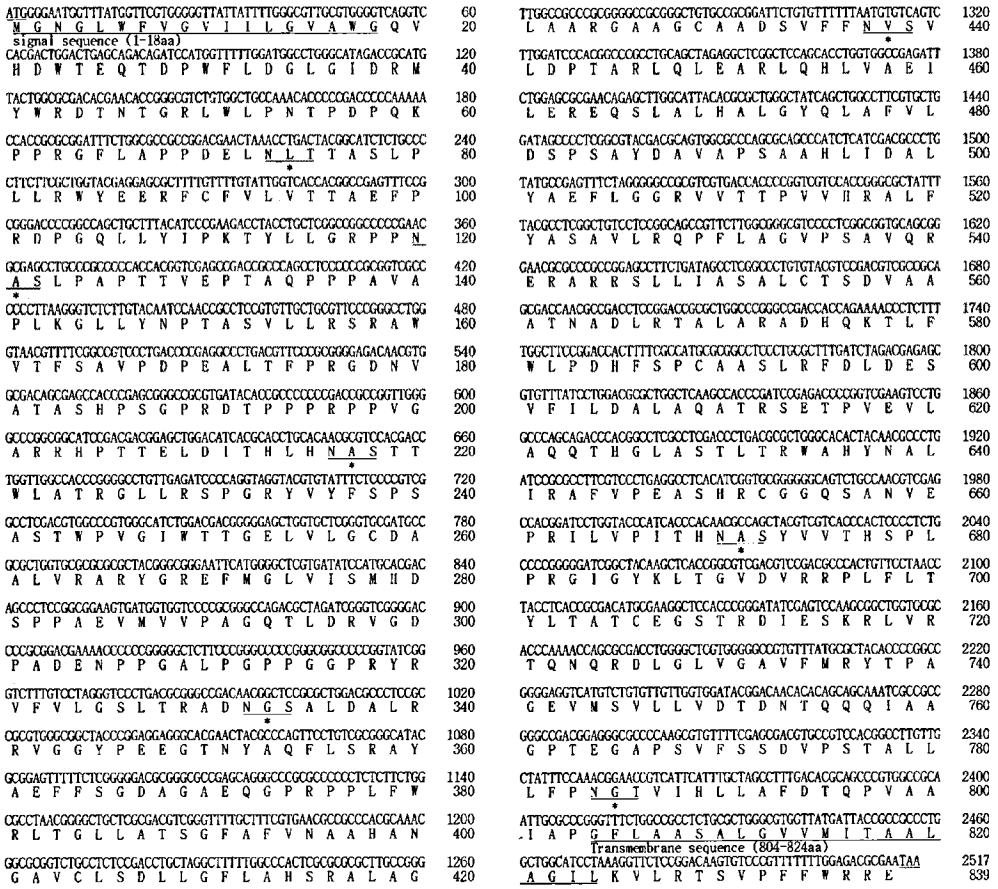

Fig. 4. Analysis of the nucleotide sequence of the ORF of the HSV-1 gH gene and the deduced amino acid sequence (GenBank accession No.AF293614). The sequence of the coding strand is given. The *gH* gene in the pBlu-GH plasmid was fragmented with restriction enzymes, subcloned, and then sequenced using the procedure of the dideoxy chain termination. Hydrophobic domains within the predicted amino acid sequence, localized by means of the Kyte and Doolittle algorithm, have been underlines (Kyte and Doolittle, 1982). Potential signal sequence cleavage site was predicted using the rules of Sipos and von Heijne (1993). The potential *N*-glycosylation sites, NXT or NXS, where X can be any amino acid (Kornfeld and Kornfeld, 1985) are underlined with asterisk.

TAA. These results were consistent with the reports of HSV-1 strain HFEM (Gompels and Minson, 1986) and HSV-1 strain 17 (McGeoch and Davison, 1986). The ORF sequence of the *gH* gene of the HSV-1 strain F in this report was compared with reported sequences of HSV strains HFEM (Gompels and Minson, 1986), SC16 (Hill *et al.*, 1975), 17 (McGeoch and Davison, 1986) and 103/65 (Buckmaster *et al.*, 1984a) (Table 1). The *gH* gene sequence was highly conserved between strains of HSV-1. Table 1 shows the differences of *gH* gene sequences in the reported HSV-1 strains, and Table 2 shows the differences within the coding region. When compared to HFEM *gH* gene sequence, 11 nucleotide differences in the HSV-1 strain F were found, of which only 5 were in the coding region. Table 2 shows the comparison of the SC16 strain 7 nucleotides, with the strain 17 five nucleotides, and with the strain 103/65 three nucleotides (Table 1), and amino acid changes according to the nucleotide changes in the late three strains.

Fig. 5 gives the hydrophobicity profile (Kyte and Doolittle, 1982) together with the positions of potential N-glycosylation sites, hydrophobic and hydrophilic amino acids, acidic amino and the basic amino residues are shown. The profile reveals the presence of two main hydrophobic regions. The first domain spans from 1 to 18 amino acid residues with a signal peptidase cleavage site, which was predicted using the rules of

Table 2. Comparison of amino acid change in the gH proteins in the different HSV strains with the F strain

Virus strains	Amino acid changed at the base pair								
	44	112	355	412	448	503	851	1717	1792
HFEM	A	M	-	A	T	-	-	-	D
SC16	-	M	T	-	T	-	-	T	D
17	-	M	-	-	-	-	V	-	D
103/65	A	M	-	-	-	G	V	-	D
F	V	I	P	S	A	D	V	A	N

Abbreviation: A, alanine; D, aspartic acid; G, glycine; I, isoleucine; M, methionine; N, asparagines; P, proline; S, serine; T, threonine; V, valine

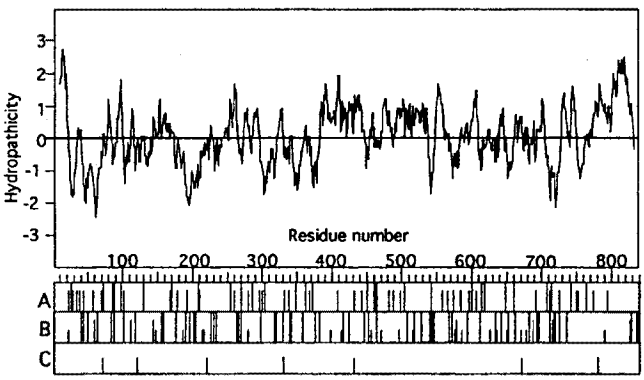

Fig. 5. Hydropathic profile of the gH polypeptide sequence predicted according to Kyte and Doolittle (1982). The mean hydropathy value is acrossed. The y axis indicates the relative hydropathicity: hydrophobic above the line and hydrophilic below the line. A, B, and C indicate the positions of acidic amino acid residues, basic amino acid residues, and potential N-glycosylation sites, respectively.

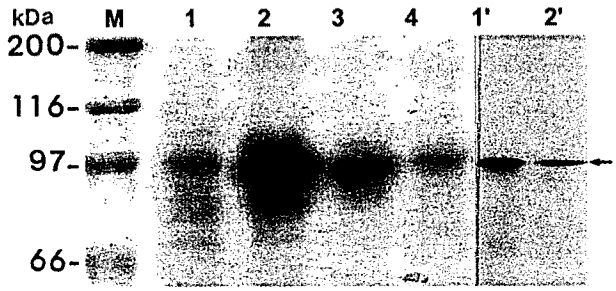

Fig. 6. PAGE of radiolabelled proteins immunoprecipitated from infected cell lysates and culture media. (A) Cells were infected with the GH1-HcNPV-1 viruses (m.o.i. = 2) for 5 days and centrifuged to separate the infected cells and cultured media. The cell pellets were lysed by a freeze-thawing, the cultured media were dialyzed, and then they were loaded for 10% SDS-PAGE. (B) At 42 h p.i. the cells were labeled with L-^{3}H-leucine for 1 h and lysated. The soluble fractions of the lysates were mixed with Mab gH and the antigen-antibody complexes were precipitated with protein A-Sepharose. Immunoprecipitates were resolved by electrophoresis on 10% SDS-PAGE, and the labeled protein was detected by PPO fluorography. Lanes 1, normal cell lysates; Lane 2, the cultured media infected with the GH1-HcNPV; Lane 3, the cell lysates infected with the virus; and Lane 4, cell lysates bearing the expression vector. Lanes 1' and 2': immunoprecipitations of the lanes 2 and 3 with the specific monoclonal antibody, respectively. Numbers at the left correspond to the positions of the molecular weight markers (in kDa).

Permian and Halvorson (1983) and von Heijne (1984) (Figs. 4 and 5). A second hydrophobic domain is the region of 21 amino acid residues from position 803 to 824 (Figs. 4 and 5). Location of this sequence near the carboxy terminus of the gH protein may serve as a membrane anchor sequence (Gompels and Minson, 1986; McGeoch and Davison, 1986; Sipos and von Heijne, 1993) and there are two polar residues, serine and threonine. Wily (1985) noted that the polar residues serine and threonine, are often found in membrane spanning stretches. The ORF was rich in alanine (107 out of a total of 838 aa) and leucine (103 out of the total of 838 aa) residues. A minimum stretch of 14 hydrophobic residues not interrupted by charged residues can span the membrane (Figs. 4 and 5) and allow correct transport of a glycoprotein to the cell surface (Adams and Rose, 1985ab).

There are seven *N*-glycosylation sites along the sequence of gH (Figs. 4 and 5). Putative *N*-glycosylation sites are at positions starting at 73, 120, 216, 332, 437, 670, and 784-Asp. The glycosylated amino acids are distributed at both N and C terminal and in the center of the protein. The presence of a putative signal sequence and a transmembrane sequence with the *N*-glycosylation sites identified on the protein sequence strongly suggests that the ORF codes for a glycoprotein.

Production and characteristics of the gH produced in insect cells The insect cells infected with the recombinant viruses GH1-HcNPV-1 produced a high level of the gX-gH fusion proteins. The production of gX-gH fusion protein in *S. frugiperda* cells infected with the recombinant viruses was detected by a SDS-PAGE analysis (Fig. 6 lanes 2 and 3) and confirmed by a radioimmunoprecipitation analysis (Fig. 6 lanes 1' and 2'). It demonstrates that a high level of a 112 kDa gX-gH fusion protein was produced in the insect cells that were infected with the recombinant viruses (Fig. 6 lane 3) and excreted into the culture media (Fig. 6 lane 2). These results demonstrate that the amounts of the secreted gH into the cultured media were about three times than the gH present in the insect cells. However, the control groups (Fig. 6 lanes 1 and 4) were not immunoprecipitated with the specific monoclonal antibody as indicated in Materials and Methods. The Pseudorabies virus gX glycoprotein accumulates in the medium of infected cells which has another 20-peptide sequence (2 kDa) at the N-terminus, and 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 plays a role for secretion of the gX-gH fusion protein from the insect cells infected with the GH1-HcNPV-1 contained the gX signal sequence. HSV-1 infected BHK cells synthesized the gH protein with a molecular weight of approximately 110 kDa (Gompels and Minson, 1989). Our result indicates that the recombinant virus GH1-HcNPV-1 expressed a high level of the glycosylated mature gH with 110 kDa in the insect cells and then accumulated in the culture media (Fig. 6 lane 2). The molecular weight of the unmodified polypeptide was found to be approximately 92,180 (838 amino acid residues). However the presence of several modifications may explain why the molecular weight of the presumptive gH (110 kDa) was estimated by gel electrophoresis. Gompels and Minson (1989) constructed a recombinant plasmid, pUG-H1 that contained HSV-1 *gH* gene using CDM8 expression vector, where gH protein was expressed in the BHK cells. Ghiasi *et al.* (1991)

Fig. 7. Immunofluorescence of gH fusion protein produced in *S. frugiperda* cells infected with GH1-HcNPV-1 recombinant virus. The virus-infected cells were fixed in acetone and treated with an anti-gH 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.

also constructed a recombinant baculovirus that contained a *Bgl*II fragment with a HSV-1 strain HFEM gH gene using the *Autographa californica* NPV expression vector, which expressed the 110 kDa gH protein within insect cells.

The production of gX-gH fusion protein in the *S. frugiperda* cells infected with the GH1-HcNPV-1 was also detected by an indirect immunofluorescence analysis using a fluorescein-conjugated gH monoclonal antibody (52S) prior to fixation. No fluorescence was seen in the cells infected with the wild-type baculovirus *lacZ*-HcNPV (data not shown), however, the GH1-HcNPV-1 infected cells exhibited high surface fluorescence with a green color (Fig. 7). The green fluorescence indicates that the gH protein was transported at the cell surface, which is consistent with the report of Ghiasi *et al.* (1991). Additionally, HCMV gH that is expressed by recombinant vaccinia virus does not appear to be localizing to the cell surface (Cranage *et al.*, 1988).

The immunogenicity of the recombinant gH was studied by immunizing mice with lysates from whole insect cells infected with GH1-HcNPV-1 as described under Materials and Methods. Three weeks after the final vaccination, mice were bled and their sera tested for HSV-1-neutralizing activity. Pooled sera were heat-inactivated and then incubated with a guinea pig complement. The sera from immunized mice exhibited a high HSV-1-neutralizing activity *in vitro* (Table 3). This suggests that the baculovirus-expressed gH can induce an immune response in mice that is directed against authentic gH. Ghiashi *et al.* (1991, 1992) obtained a similar result, where antibodies raised in mice to recombinants recognized viral gH and neutralized the infectivity of HSV-1 *in vitro*.

In conclusion, these results demonstrate that the gH gene was localized in the 6.41 kb *Bgl*II fragment of HSV-1 (F)

Table 3. Neutralizing antibody titers in mice vaccinated with a GH1-HcNPV-1

Immunogens	No. of mice tested	50% neutralization titer of antisera
GH1-HcNPV-1	10	<1: 128
HSV-1 KOS	10	<1: 1024
Mock (HcNPV)	10	<1: 8

genome and the GH-HcNPV recombinant virus produce a mature gX-gH fusion protein by gene transfer in insect cells and the fusion proteins were accumulated in the medium. This indicate the utility of the HcNPV-insect cell system for producing and characterizing eukaryotic proteins. The 833 aa ORF of this gene encoded a polypeptide of 110 kDa that had all the characteristics of a glycoprotein. The deduced amino acid sequence showed a high level of similarity with the reported HSV-1 gHs sequences. Although the level of viral latency was not measured in this study, our results show that a high level of neutralizing antibodies production may protect mice against HSV and this particular recombinant protein may be useful in the development of a subunit vaccine.

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