J. Microbiol. Biotechnol. (1999), **9**(5), 541–547

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Constructions of a Transfer Vector Containing the gX Signal Sequence of Pseudorabies Virus and a Recombinant Baculovirus

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Received: March 12, 1999

Abstract Constructions of a transfer vector and a recombinant baculovirus using the thymidine kinase gene of the *Herpes simplex* virus type 1 strain F (HSV-1) were carried out. Newly cloned transfer vector, pHcgXIIIB, was constructed by insertion of the glycoprotein gX gene signal peptide sequence of Pseudorabies virus into the baculovirus vector pHcEV-IV. The gX sequence was inserted just downstream from the promoter for the polyhedrin gene of the *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV). HSV-1 thymidine kinase (*tk*) gene (1.131 kb) was used as a candidate gene for transferring into the baculovirus expression system. The *tk* gene was inserted into a *Bam*HI site downstream from the gX sequence-promoter for the polyhedrin gene in the pHcgXIIIB transfer vector and was transferred into the infectious *lacZ*-HcNPV expression vector. Recombinant virus was isolated and was named gX-TK-HcNPV. The recombinant virus produced a 45 kDa gX-TK fusion protein in *Spodoptera frugiperda* cells, which was confirmed by Western blot analysis. Microscopic examination of gX-TK-HcNPV-infected cells revealed normal multiplication. Fluorescent antibody staining indicated that the gX-TK fusion protein was present in the cytoplasm. These results indicated that the transfer vector successfully transferred the gX-*tk* gene into the baculovirus expression system.

Key words: Herpesvirus thymidine kinase, Pseudorabies glycoprotein X, baculovirus vector, recombinant protein

Previously, a Baculovirus transfer vector with the *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) was constructed [14]. It can be improved by adding the type of promoter used or by providing a signal sequence for secretion or expression as a fusion protein. The choice of transfer plasmid will significantly influence methods to identify the recombinant and may also dictate the nature of the parent virus used in the allelic replacement event [22]. Pseudorabies virus glycoprotein gX (498 amino acids) accumulates in the medium of infected cells, and has another signal 20 peptide sequence at the N-terminus [23]. This signal sequence might play an important role in the accumulation of the gX in the medium. Therefore, the gX signal sequence [23] was previously used to improve the transfer vector, pHcEV-IV [14].

The thymidine kinase (*tk*) gene of *Herpes simplex* virus (HSV-1) is an early gene [11] and thymidine kinase (TK) phosphorylates purine pentosides and a wide varieties of nucleoside analogues more efficiently than other cellular kinases [4, 6]. Therefore, we used the *tk* gene as a candidate gene to be transferred into the Baculovirus expression system [14]. Baculovirus vectors have been recognized to be very useful in achieving a high-level expression of a variety of foreign genes and also in having the advantage over transformed cell lines in that it can be expressed under a variety of different conditions. This was achieved by changing parameters such as the host cell or the multiplicity of infection [8, 11, 13, 14, 15, 31]. Here, the construction of baculovirus, HcNPV-based transfer vector, was made containing the gX-TK coding sequence necessary to conduct studies on the effects of high-level expression of gX-TK in insect cells. In addition, the TK-expressing HcNPV vector was also used for the *in vivo* studies on immunological properties.

Materials and Methods

Virus and Cell

The *lacZ-Hyphantria cunea* nuclear polyhedrosis virus (*lacZ*-HcNPV) strain 1 [14] and the wild-type HcNPV HL-2 strain [12] were used for cloning the *tk* gene of HSV-1, and propagated in *Spodoptera frugiperda* cells (IPLB-

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SF-21) [29] at 28°C in TC-100 medium (Gibco, Middleton, WI, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Gibco), 0.2% tryptose broth powder, and 50 μg/ml gentamycin (Gibco). The virus was titrated for infectivity as described by Lee *et al.* [12]. Virus inoculum was extracellular nonoccluded virus (NOV) derived from a cell culture medium [12, 18].

Plasmids and Bacteria

Escherichia coli XL1-Blue/pBac-TK clone [17], which contained a full-length TK coding sequence from HSV-1 strain F, was used for the *tk* gene source. *E. coli* M13KO7/ pSL1190-gXp-SV40 poly-A(Amersham Pharmacia Biotech Korea, Seoul) used for a source of the gX signal sequence was obtained from Dr. J. Y. Song (Virology Division, National Veterinary Research and Quarantine Service, Kyunggi-do, 430-016, Korea). *E. coli* M15/pQE-TK was also used for the TK protein source [17]. *E. coli* were maintained in LB-broth (Difco Laboratories, Detroit, U.S.A.).

Primers and Probe

Synthetic oligomers (primers), k-1 and k-2, were deduced from sequences of the HSV-1 strain CL101 *tk* gene [30]. All primers were synthesized at the Korea Biotech. Inc. (Taejon, Korea) (Table 1). The primer k-1 at the 5' end contains a *Bam*HI site and start codon ATG. The primer k-2 at the 3' end contained an *Eco*RI site and stop codon TGA. Clone pHLA-12 clone [11] was used as a probe DNA for the *tk* gene sequence.

Restriction Enzymes

All restriction endonucleases, calf intestinal alkaline phosphatase, T4 DNA ligase, and Taq DNA polymerase were obtained from Boehringer Mannheim (Indianapolis, U.S.A.) and Promega (Arlington Heights, U.S.A.).

Radioisotopes and Antibodies

[α-^{35}S]dATP for nucleotide sequencing was obtained from Amersham (Buckinghamshire, U.K.). The secondary antibodies for immunoblotting and immunofluorescence assays (IFA) were obtained from Boehringer Mannheim Biochemicals.

Purification of Virus and Viral DNA

S. frugiperda cells (5×10^6 cells per 25 cm^2 flask) were inoculated with *lac*Z-HcNPV at the multiplicity of infection (m.o.i.) of 5. The cells were incubated at 28°C for 1 h while shaking every 15 min. Five milliliters of TC-100 medium containing 5% FBS was then added to the dishes and incubated at 28°C for 72 h. Nonoccluded virions and viral DNAs were purified by the procedure described by Lee [12] and Lee and Lee [19].

Table 1. List of primers.

Primer Sequence
k-1 5'-TTTGGCAAGGGATCCGTAGAAGCGCGT**ATG-3'**
k-2 5'-CCCGAAT**TC**AGTTAGCCTCCCCCACTCCCC-3'

The *Bam*HI site is underlined and the ATG translation start codon is boldfaced in primer k-1. The *Eco*RI site is underlined and nucleotide sequences complementary to the TGA termination codon is boldfaced in primer k-2.

Plasmid Isolation

E. coli containing recombinant plasmids were cultured in LB broth (Difco) at 37°C. The plasmid DNA was then purified according to the procedure described by Birnboim and Doly [1].

Restriction Enzyme Digestions and Agarose Gel Electrophoresis

All restriction endonuclease digestions were performed according to the manufacturer's instructions. HcNPV DNA genome and vector DNAs were digested and electrophoresed on 1.0% agarose gel. The molecular size of each DNA fragment was determined by comparing their mobility with *Hin*dIII-digested phage λ DNA fragments. The reactions were terminated by adding 1/10 volume of the stop solution [24]. Details of procedures for gel electrophoresis and visualization of the DNA fragments have been described by Lee *et al.* [16]. DNA fragments were fractionated and purified in 1.0% low melting temperature-agarose gels containing Tris-borate and ethidium bromide [15, 16].

Cloning and Transformation

Cloning was carried out by mixing 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 50 μl of the solution was reacted for 18 h at 14°C [15, 24]. The progress of the reaction was examined by 1.0% agarose gel electrophoresis [16]. The *E. coli* competent cells were prepared and transformed by the method of Mandel and Higa [20].

Construction of Transfer Vector pHcgXIIIB

Pseudorabies virus (Aujeszky's disease: ADV) gX signal peptide sequence- SV40-poly A sequence of the pSL1190-gXp-SV40 poly A recombinant plasmid was cleaved out with *Bgl*II, cloned into the *Bam*HI site of the pHcEV-IV vector, and was named pHcgX1 plasmid. The pHcgX1 was treated with *Sma*I and *Nco*I, and again with S1 enzymes to remove the restriction enzyme sites from the MCS, which was self-ligated, and the final product was named pHcgXIIIB recombinant vector (Fig. 1).

Construction and Selection of Recombinant Virus

The *S. frugiperda* cells were cotransfected with linearized *lac*Z-HcNPV DNA and pHc-TK plasmid DNA, and then

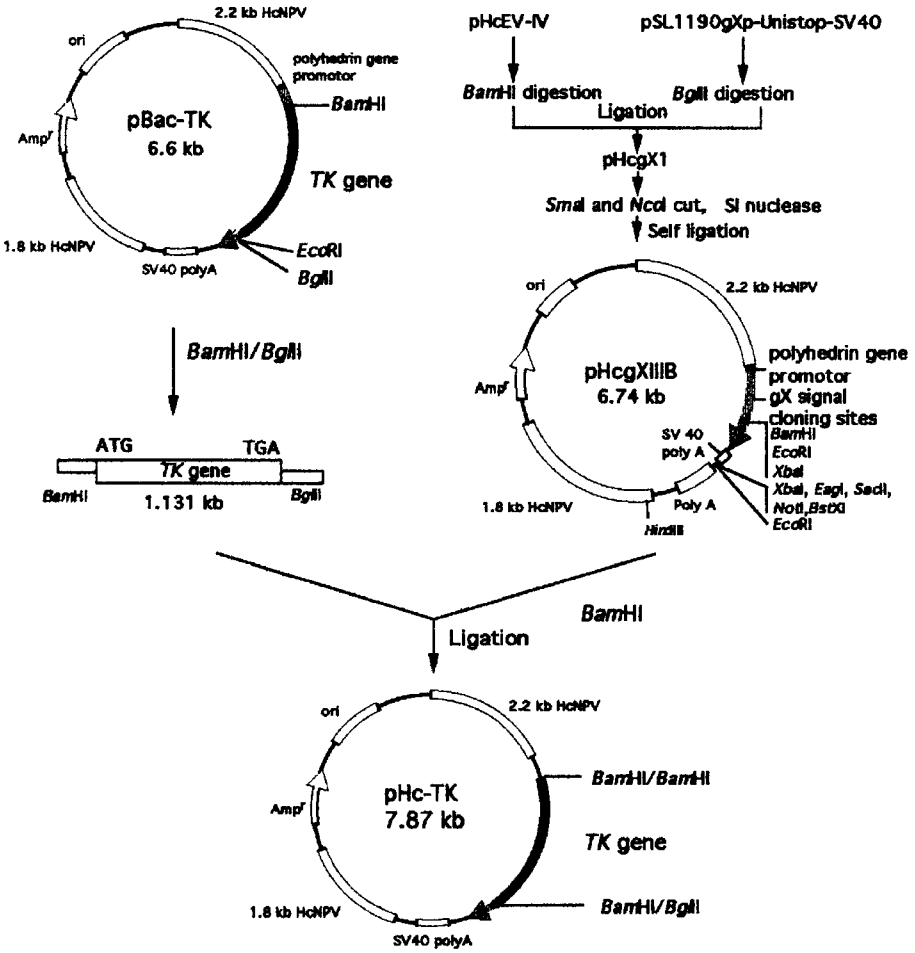

Fig. 1. Construction of transfer vector pHcgXIIIB and pHc-TK recombinant plasmid. The *tk* gene sequence in the pBac-TK plasmid was transferred into the *Bam*HI site of pHcgXIIIB vector and named pHc-TK plasmid.

putative recombinant viruses were constructed as previously described by Lee *et al.* [13, 14]. Recombinant viruses containing the combined gX-*tk* gene in their genomes were selected by plaque assay with X-gal as described by Lee *et al.* [13, 14, 15].

Analyses of SDS-PAGE and Western Blot

TK proteins were fractionated on 10% polyacrylamide gels and analyzed by Western blot as described by Bollag *et al.* [2].

Amplification of DNAs and Southern Blot

Polymerase chain reaction (PCR) amplification of DNA was carried out by the method of Saiki *et al.* [26] and Chung *et al.* [5]. Southern blot analysis was performed by using the procedure described by Southern [27].

Nucleotide Sequencing

Dideoxy chain termination sequencing [25] was performed on 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. These fragments were then sequenced with a 7-deaza-dGTP sequencing kit and Sequenase version 2.0 T7 DNA polymerase (United State Biochemical, Cleaveland, OH), by the use of pBluescript T3 primer, T7 DNA primer, and M13 reverse primer.

Preparation of TK Polyclonal Antiserum

TK protein antigen, which was produced by the *E. coli* M15/pQE-TK clone [17], was purified by the metal affinity chromatography with the procedure described by Hochuli *et al.* [9] and Lee *et al.* [13]. Protein concentration was measured as described by Bradford [3]. Mice were immunized with 0.5-50 μg TK protein in complete Freund's adjuvant (Gibco) by peritoneal cavity injection. After 3 weeks, one booster injection was performed with 0.5-50 μg of the protein in incomplete Freund's adjuvant (Gibco). To obtain an antiserum after 10-14 days, a blood sample was collected from the heart, incubated at 4°C for 18 h, and centrifuged at 4,000 ×g for 10 min. Then, the supernatant was stored at -20°C.

Immunofluorescence Assay

For detection of the proteins in cells, immunofluorescence assay (IFA) was carried out using a modification of the procedure described by William *et al.* [31]. *S. frugiperda* cells (2×10^6 cells per dish) were seeded on a glass cover slip (18×18 mm) in petri dishes (60 mm^2), infected with recombinant baculoviruses at a m.o.i. of 10 pfu/cell, and incubated for 72 h. The infected cells grown on the glass cover slip were fixed with cold acetone (-10°C) for 10-20 min. The fixed cells were washed in PBS (pH 8.0) for 10 min, incubated for 0.5-1 h at room temperature with TK polyclonal antibodies diluted 1:500 in PBS, and subsequently washed three times with 0.5% BSA in PBS each for 15 min. The cells were incubated for 30-60 min with fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G (Boehringer Mannheim Biochemicals) diluted 1:30 in PBS, 0.5% BSA. The cells were washed three times with PBS for 15 min, mounted in mounting buffer (1 glycerol: 5 PBS), and then examined at 400× or 1000× magnifications under a fluorescent microscope with a Leitz 12 filter allowing illumination at 450-410 nm wavelength.

RESULTS AND DISCUSSION

Construction of Transfer Vector pHcgXIIIB and Recombinant Virus

A previously constructed baculovirus *H. cunea* nuclear polyhedrosis virus (HcNPV) transfer vector, pHcEV-IV (6.7 kb) [14], was modified by insertion of the 400 bp Pseudorabies virus glycoprotein gX gene signal peptide sequence from the pSL1190-gXp-SV40 vector, and was named pHcgXIIIB transfer vector plasmid (6.74 kb) (Fig. 1). The insertion of the gX signal sequence in pHcgXIIIB was confirmed by DNA sequence analysis (Fig. 2). The gX gene signal sequence was correctly oriented in front of the downstream from the promoter for the HcNPV polyhedrin gene. One *Bam*HI site located just downstream of the gX signal sequence was used specifically for cloning of transferring genes. The gX signal peptide sequence consisted of 20 amino acid residues (Fig. 2). The new transfer vector, pHcgXIIIB, was used to transfer the candidate *tk* gene into the *lac*Z-HcNPV expression vector.

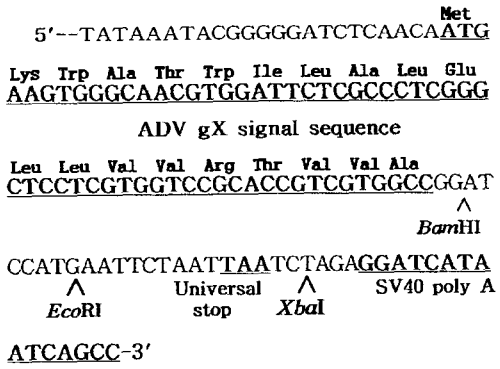

Fig. 2. Confirmation of insertion of the gX gene signal sequence in the pHcgXIIIB vector by partial DNA sequencing.
The sequences are underlined. The gX signal peptide sequence was deduced from the gX gene sequence.

The *tk* gene sequence (1.131 kb) from HSV-1 in the pBac-TK clone [17] was digested with *Bam*HI and *Bgl*II, inserted into the *Bam*HI site downstream from the gX signal sequence in the transfer vector pHcgXIIIB, and named pHc-TK plasmid (7.87 kb) (Fig. 1). The insertion of the gene sequences was confirmed by the restriction pattern (Fig. 3A), Southern blot analysis (Fig. 3B, lane 4), and partial DNA sequencing (Fig. 4). The pHc-TK plasmid was cleaved into three fragments, 0.5, 1.5, and 5.97 kb (Fig. 3A, lane 4), and the pBac-TK was cleaved by *Bam*HI and *Eco*RI double digestion into two fragments, 5.1 and 1.5 kb (Fig. 3A, lane 5), respectively. The PCR products of the 1.13 kb and 1.5 kb fragments were hybridized with the probe (Fig. 3, lanes 3, 4, 5). These results indicated that the gX-*tk* gene was transferred into the pHc-TK recombinant plasmid. The right-oriented insertion of the *tk* gene in the recombinant plasmid pHc-TK was confirmed by partial DNA sequencing of the ligation site (Fig. 4). The *Bam*HI cloning site of the

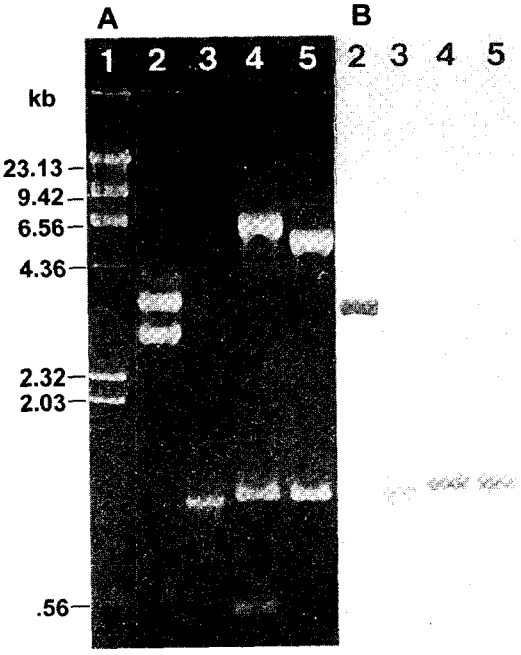

Fig. 3. Agarose gel electrophoresis patterns (A) and Southern blot analysis (B) of the *tk* gene-containing plasmids.
Lanes 1, phage λ DNA digested with *Hin*dIII; 2, pHLA-12 probe DNAs digested with *Bam*HI; 3, PCR product (1.131 kb) of *tk* gene of HSV-1; 4, pHc-TK DNAs double-digested with *Bam*HI and *Eco*RI; 5, pBac-TK DNAs double-digested with *Bam*HI and *Eco*RI. B is the Southern blot of the electrophoregram A with the probe, pHLA-12 clone.

gX ↔ *Bam*HI

CGTCGTGGCCGGATCCGTAGTTGC

GCGT[ATG]CTTCGTACCCCTGCCATC

tk gene →

Fig. 4. Confirmation of the *tk* gene insertion in front of the gX signal sequence in the pHc-TK plasmid by partial DNA sequencing.

The sequence near the translation start codon of the *tk* gene and the gX in the pHc-TK clone were sequenced. The gX-*Bam*HI ligation site (outlined letters)-ATG codon (black box) for TK translation- partial sequence of the *tk* gene in the pHcgXIIIB vector are shown.

transfer vector pHcgXIIIB-ATG translation start codon of the *tk* gene are shown in Fig. 4. These results indicated that the gX-*tk* gene was correctly transferred into the transfer vector.

To construct recombinant virus, the transfer vector pHc-TK DNA containing the *tk* gene was cotransfected with infectious *lac*Z-HcNPV DNA digested with *Bsu*36I in *S. frugiperda* cells by the lipofectin-mediated transfection as previously described by Lee *et al.* [14, 15]. Then, gX-TK-HcNPV recombinant viruses were isolated by plaque assay with X-gal (Fig. 5). These were plaque-purified to genetic homogeneity and further multiplied for the use as viral stocks. Virus plaques (two to four mm in diameter) appeared after 3 to 4 days post infection (p.i.). Several plaques were selected and viral DNAs from these plaques were analyzed by restriction digestion. All of the virus isolates contained the DNA structure outlined in Fig. 3. One large plaque, named as TK-HcNPV-1, was used for further experiments. The titer of the recombinant virus was similar to those of wild-type HcNPV (10^8 pfu/ml). The infected *S. frugiperda* cells in the plaques did not form any polyhedral inclusion bodies (Fig. 5). These results are similar to the reports by Lee *et al.* [14, 15].

The insertion of the combined gX-*tk* gene into the recombinant virus genome was confirmed by PCR (Fig. 6). The viral template DNAs extracted from the insect cells

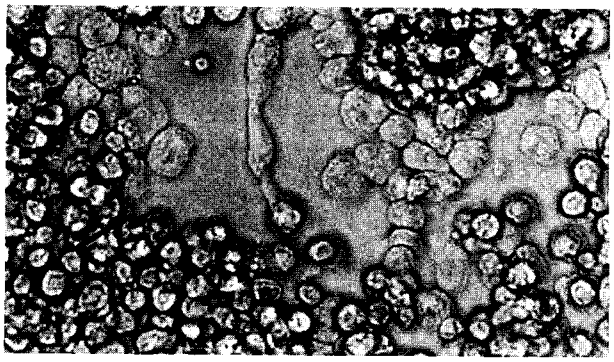

Fig. 5. Micrograph of plaques formed by the recombinant virus gX-TK-HcNPV-1 on the *S. frugiperda* cell monolayer.

The cells in the plaques formed by the recombinant virus were swollen and did not form polyhedra.

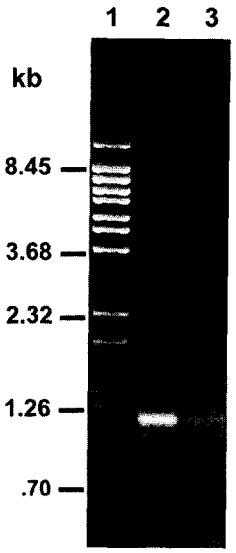

Fig. 6. PCR-detection of the TK gene insertion in the gX-TK-HcNPV-1 recombinant virus.

Lanes 1, phage λ DNA digested with *Bst*EII; 2, *tk* gene sequence of the HSV-1 strain F genome; 3, *tk* gene sequence of the gX-TK-HcNPV-1 genome obtained by PCR, respectively.

infected with the gX-TK-HcNPV-1 or HSV-1 [7] were amplified by PCR using the primers. The PCR products were then electrophoresed on agarose gel and compared. Both fragments amplified from the gX-TK-HcNPV-1 recombinant virus DNA and the HSV-1 DNA are shown in Fig. 6 (lanes 1 and 2). Lane 1 represents the PCR product of the HSV-1 and lane 2 that of the TK-HcNPV. There are approximately 1.13 kb DNA fragments corresponding to the *tk* gene sequence [17]. These results demonstrated that the combined gX-*tk* gene sequence cloned in the transfer vector was transferred into the recombinant virus gX-TK-HcNPV-1.

Expression of the gX-TK Fusion Protein in Insect Cells

S. frugiperda cells infected with the gX-TK-HcNPV-1 recombinant virus underwent the virus cytopathic effect and various morphologic alterations (Fig. 5). The entire infection process, including viral multiplication, viral assembly, and partial cell lysis, was completed by 72 h p.i. (data not shown). The titer of recombinant TK-HcNPV-infected cells reached a maximum of 3×10^8 pfu/ml of medium. Thus, the insertion of the *tk* gene into the polyhedrin gene locus had no major effect on replication of these recombinant viruses.

Mock-infected *S. frugiperda* cells and the cells infected with the TK-HcNPV were analyzed using a 10% SDS-PAGE and Western blot (Fig. 7). The 45 kDa band of thymidine kinase (TK) (43 kDa)-gX signal peptide (2 kDa) fusion protein appeared at lane 2 (Fig. 7) and was confirmed by Western blot analysis (Fig. 7, lane 2'). These results differed from the reports of Munyon *et al.* [21] and

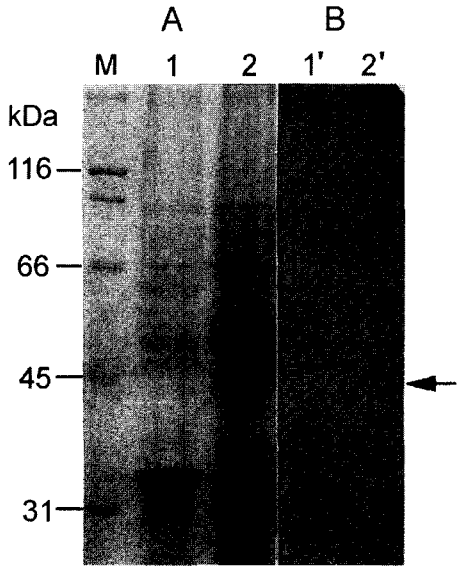

Fig. 7. Detection of gX-TK fusion proteins produced by gX-TK-HcNPV-1.

The gX-TK fusion proteins produced in *S. frugiperda* cells infected with gX-TK-HcNPV-1 were analyzed on 10% SDS-PAGE and confirmed using antibodies by Western blot analysis. Lanes M, standard molecular markers; 1, lysate of cells infected with wild-type HcNPV; 2, lysate of the cells infected with the gX-TK-HcNPV-1. Panel B is the Southern blot results of the panal A. The arrow indicates the ca. 45 kDa gX-TK fusion protein.

Lee *et al.* [17] in that the molecular weight of the TK of HSV-1 was 43 kDa. Lane 1a is the negative control of wild-type HcNPV in comparison with the gX-TK-HcNPV clone, wherein the TK did not appear, but the polyhedrin band was apparent in the cell extracts infected with the wild-type HcNPV. The production of gX-TK protein in the cells infected with the recombinant virus was detected by indirect immunofluorescence analysis. To examine the subcellular distribution of gX-TK in gX-TK-HcNPV-infected cells, the cells were fixed and stained with mouse polyclonal antibodies and fluoresceinated sheep anti-mouse antibodies (Fig. 8). The gX-TK-HcNPV-infected cells showed evenly high fluorescence with a green color (Fig. 8). No immunofluorescence was seen in the cells infected with the wild-type *lac*Z-HcNPV. This fluorescent green color suggested that a high level of the gX-TK protein was produced and evenly distributed in the cell cytoplasms. It has previously been shown that TK proteins were present in the soluble cytoplasmic fraction [17], which is further supported by the presently described immunofluorescence analysis.

Fig. 8. Detection of the intracellular TK protein produced in *S. frugiperda* cells infected with the TK-HcNPV-1 by immunofluorescence assay.

The cells infected with the TK-HcNPV-1 were fixed with acetone, incubated with anti-TK polyclonal antibody followed by fluorescein-conjugated sheep anti-mouse antibody and examined by fluorescence microscopy. The recombinant baculovirus-infected cells became totally fluorescent. Arrows indicate the fluorescent cells with a green color.

In conclusion, this report demonstrates the construction of a new transfer vector, which transfered a candidate *tk* gene into the *lac*Z-HcNPV expression vector, and expressed the *gX-tk* gene in the insect cells infected by the recombinant virus. These results show that the transferring of a foreign gene by the transfer vector from other sources into the baculovirus expression vector could produce a high level of protein effectively.

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