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Expression of Bovine Growth Hormone Gene in a Baculovirus, *Hyphantria cunea* Nuclear Polyhedrosis Virus

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=Abstract=

Bovine growth hormone (bGH) gene was expressed in an insect *Spodoptera frugiperda* cell line using a Baculovirus, *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV). The bGH gene in pbGH plasmid was sequenced and amplified by PCR technique with two primers containing *NcoI* sites. The bGH gene consisted of 654 bp (217 amino acid residues), the 5'-untranslated region of the cloned bGH cDNA contains 56 bp, and the 3'-untranslated region contains 145 bp and two pallindromic regions. The amplified bGH gene DNA fragment (654 bp) was inserted into the *NcoI* site of the pHcEVII vector, which was named pHcbGH. The pHcbGH transfer vector DNA and the wild type HcNPV DNA were cotransfected into *S. frugiperda* cells to construct a recombinant virus. Eight recombinant viruses were selected and named HcbGH. One clone, HcbGH-4-1 showed largest plaque size, therefore the recombinant virus was further studied. The multiplication pattern of the recombinant HcbGH-4-1 was similar to that of the wild type HcNPV. The bGH gene DNA in the HcbGH-4-1 recombinant was confirmed by Southern blot hybridization. The amount of the bGH (217 amino acid residues, 21 kDa) produced in *S. frugiperda* cells infected with the HcbGH-4-1 recombinant was approximately 5.5 ng per ml (10^6 cells) by radioimmunoassay.

Key Words: Baculovirus vector, Bovine growth hormone, *Hyphantria cunea* nuclear polyhedrosis virus

INTRODUCTION

Baculoviruses are pathogens that cause fatal disease in insects and are used to control insect pests in agriculture and forestry [6], and has a double stranded DNA genome with molecular weight of 80 mega Da [3, 10, 11]. They are also used as vectors for the expression of foreign genes in insect cells [1, 4, 9, 19, 20, 21, 23, 24, 25, 27, 31]. Baculovirus expression vectors are based upon the allelic replacement of the polyhedrin gene by a foreign gene and in these systems, foreign genes placed under the control of the strong polyhedrin promoter of baculovirus are usually expressed at high levels in cultured insect cells [9, 20, 21, 25]. The recombinant proteins appear to be similar to their authentic counterparts with the exception of the extent of their glycosylation. The recombinant proteins can be effectively used as diagnostics, therapeutics, and vaccines

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[19, 27].

One of baculovirus, *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) was isolated and replicated in the nucleus of *Spodoptera frugiperda* cell line infected by the virions [7]. Physical mapping of the HcNPV genome DNA was done by restriction enzymes *Bam*HI and *Sma*I [11], and then restriction patterns of the genome were analyzed with eight restriction enzymes [10]. Also the polyhedrin of HcNPV was cloned [15] and sequenced [14, 20]. Based on this information, a vector for expression and transfer foreign genes was constructed [20]. Therefore this study has been underaken to use the Baculovirus vector for cloning and expression.

In this study, a candidate gene for incorporation into a baculovirus, HcNPV was a bovine growth hormone gene in pbGH recombinant plasmid. The recombination of the gene into the vectors and the host HcNPV, and the expression of the gene by the recombinant virus in an insect cell, *S. frugiperda* cell line, were investigated.

MATERIALS AND METHODS

Virus, cell line and medium

The plaque-purified clone HL-2 of *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) was used [7]. The virus inoculum used was extracellular nonoccluded virus (NOV) derived from a cell culture medium [7, 12, 13, 16]. *Spodoptera frugiperda* cell line (IPLB-SP-21) [30] was obtained from L.K. Miller (University of Georgia, USA). The cells were propagated at 27℃ in TNM-FH medium (K.C. Biological, USA) supplemented with 0.26% tryptose broth (named as TNM-FH-T), 10% fetal bovine serum (GIBCO), 100 units of penicillin and 100 μg of streptomycin per ml. The cells routinely maintained in TNM-FH-T medium at 27℃ and passed every 4 days. For experiments requiring large numbers of cells, the cells were cultured in 200 to 300 ml suspension at 27℃.

Plasmids, probe DNA and primers

pbGH recombinant plasmid with bGH gene in *E. coli* XL-1 [8] and pHcEVII vector in *E. coli* JM83 [20] were used. To verify the presence of the bGH gene segment in the recombinant virus, Southern hybridization [26] was performed with the probe DNA of bGH gene [8].

Two primers (primer-1; 5'-GTGGACACCATGGCAGCTATG-3' and primer-2; 5'-GGAAGATCAACGGTCGGTACCCAAC-3') were synthesized at Korea Basic Science Center (Seoul) and used as primers for polymerase chain reaction (PCR) and for site directed mutagenesis of translation start and termination regions of bovine growth hormone (bGH) gene. The two primers were designed for *Nco*I enzyme sites at the both end regions of the bGH gene.

Virus multiplication and DNA purification

Wild type virus, *H. cunea* NPV and recombinant viruses were multiplied and observed by the description of Lee et al., [7, 16]. Virus DNAs were purified as described by Lee et al., [9].

Plasmid isolation

E. coli containing recombinant plasmids was cultured in LB broth (DIFCO) at 37℃ and then the plasmid DNA was purified as described by the Birnboim and Doly [2].

Restriction digestion and agarose gel electrophoresis

All restriction endonuclease digestions were performed according to the manufacturer's instructions. Details of agarose gel electrophoresis and visualization of the DNA fragments have been described by Lee et al., [11]. The extent of digestion was determined by agarose gel electrophoresis of the sample. The enzyme digested DNA was electrophoresed on 1.0% agarose gel and the molecular size of each DNA fragment was determined by comparing its mobility with *Hind*III-digested phage λ DNA

fragments. The bGH gene fragment was mapped by restriction enzymes [11].

Elution of DNA from agarose gels

DNAs from low melting agarose gel was eluted by the slightly modified procedure of Weislander [32]. Restriction DNA fragments were fractionated in 1.0% low melting temperature-agarose gels containing tris-borate and ethidium bromide. The bands were identified using 300 nm long wavelength ultraviolet light and excised from the gel. The sliced gel was melted at 65℃, diluted to 0.2% agarose in 100 mM tris-HCl (pH 8), cooled to 37℃, and mixed with phenol for 25 min at 37℃. The organic and aqueous layer was reextracted briefly with phenol/chloroform (1:1) in a TE buffer (10 mM Tris-Cl, 1.0 mM EDTA). After recentrifugation at 10,000 x g the DNA was precipitated with ethanol, pelleted and dissolved in the TE buffer.

DNA sequence analysis

The nucleotide sequence of the bovine growth hormone (bGH) gene in the pbGH recombinant plasmid was determined by the dideoxynucleotide chain termination method [22]. Squencing strategy is illustrated in Fig. 1.

Site-directed mutagenesis

For inserting NcoI enzyme site into both ends of the bGH gene in the pbGH recombinant plasmid and for amplifying the gene sequence, site-directed mutagenesis were performed using the previous PCR technique [20].

Cloning of bGH gene into pHcEVII vector

The amplified DNA fragment of the bGH gene was digested with *Nco*I enzyme and ligated into the NcoI site of the pHcEVII vector [20] (Fig. 3) and transformed as described by the Mandel and Higa [17]. This recombinant plasmid was named pHcbGH, which was redigested with *Nco*I enzyme for confirmation of cloning and its size. And then its orientation was confirmed by the digestions with *Sma*I and *Pvu*II enzymes.

Construction of recombinant virus with bGH gene

Construction of a recombinant virus with HcNPV was carried out as described by Lee et al., [9]. Construction scheme of a recombinant baculovirus containing the bGH gene was illustrated in Fig. 5. Using lipofectin technique [5], pHcbGH recombinant DNA containing the bGH gene (Fig. 5) was transfected into *S. frugiperda* cells along with the wild type HcNPV DNA, and then followed the procedure.

Isolation of recombinant virus by plaque assay

A plaque assay was carried out for HcNPV and recombinant virus on the *S. frugiperda*

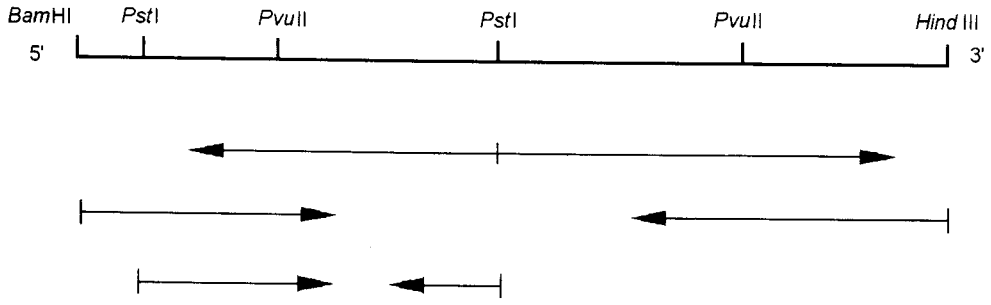

Fig. 1. A physical map and sequencing strategy of the bovine growth hormone cDNA. Three restriction sites are on the *Bam*HI-*Hind*III fragment DNA. The arrows indicate the sequencing directions and the length of the sequenced DNA.

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GATCCCAGGACCCAGTTCACCAGACGACTCAGGGTCCTGTGGACAGCTCACCAGCTATG ATG GCT
                                                          met met ala

GCA GCC CCC GGA ACC TCC CTG CTC CTG GCT TTC GCC CTG CTC TGC CTG CCC
ala gly pro arg thr ser leu leu leu ala phe ala leu leu cys leu pro

TGG ACT CAG GTG GTG GGC GCC TTC CCA GCC ATG TCC TTG TCC GGC CTG TTT
trp thr gln val val gly ala phe pro ala met ser leu ser gly leu phe

GCC AAC GCT GTG CTC CGG GCT CAG CAC CTG CAT CAG CTG GCT GCT GAC ACC
ala asn ala val leu arg ala gln his leu his gln leu ala ala asp thr

TTC AAA GAG TTT GAG CGC ACC TAC ATC CCG GAG GGA CAG AGA TAC TCC ATC
phe lys glu phe glu arg thr tyr ile pro glu gly gln arg tyr ser ile

CAG AAC ACC CAG GTT GCC TTC TGC TTC TCT GAA ACC ATC CCG GCC CCC ACG
gln asn thr gln val ala phe cys phe ser glu thr ile pro ala pro thr

GGC AAG AAT GAG GCC CAG CAG AAA TCA GAC TTG GAG CTG CTT CGC ATC TCA
gly lys asn glu ala gln gln lys ser asp leu glu leu leu arg ile ser

CTG CTC CTC ATC CAG TCG TGG CTT GGG CCC CTG CAG TTC CTC AGC AGA GTC
leu leu leu ile gln ser trp leu gly pro leu gln phe leu ser arg val

TTC ACC AAC AGC TTG GTG TTT GGC ACC TCG GAC CGT GTC TAT GAG AAG CTG
phe thr asn ser leu val phe gly thr ser asp arg val tyr glu lys leu

AAG GAC CTG GAG GAA GGC ATC CTG GCC CTG ATG CGG GAG CTG GAA GAT GGC
lys asp leu glu glu gly ile leu ala leu met arg glu leu glu asp gly

ACC CCC CGG GCT GGG CAG ATC CTC AAG CAG ACC TAT GAC AAA TTT GAC ACA
thr pro arg ala gly gln ile leu lys gln thr tyr asp lys phe asp thr

AAC ATG CGC AGT GAC GAC GCG CTG CTC AAG AAC TAC GGT CTG CTC TCC TGC
asn met arg ser asp asp ala leu leu lys asn tyr gly leu leu ser cys

TTC CGG AAG GAC CTG CAT AAG ACG GAG ACG TAC CTG AGG GTC ATG AAG TGC
phe arg lys asp leu his lys thr glu thr tyr leu arg val met lys cys

CGC CGC TTC GGG GAG GCC AGC TGT GCC TTC TAG TTGCCAGCCATCTGTTGTTTGCC
arg arg phe gly glu ala ser cys ala phe am

CCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTCCTAAATAAAATGAGGAA

ATTGCATCGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACCTGCAGCCAAGCT
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Fig. 2. Nucleotide sequence of bovine growth hormone cDNA (mRNA) and its derived amino acid sequence as determined by the nucleotide sequence of the cDNA contained in pbGH. The boxes indicate translation start codon, ATG and termination codon, TAG. The underlined bases at 3'-untranslated sequence indicate the two pallindromic regions.

cell line as described by Lee et al., [9]. By the plaque assay recombinant virus clones were isolated.

Southern blot

For Southern analysis, recombinant viral DNA preparations were completely digested with *Eco*RI. The probe DNA was labelled *in vitro* by ECL (enhanced chemilluminescence) procedure [29], using 20 μl (300 ng) of bGH gene cDNA, 20 μl of horse radish peroxidase and 20 μl of glutaraldehyde. The total mixture (60 μl) was incubated for 10 minutes at 37℃, and held on ice for 10-15 minutes. DNA fragments of recombinant virus on the gels were transferred on Hybond-N^+ filter (Amersham)

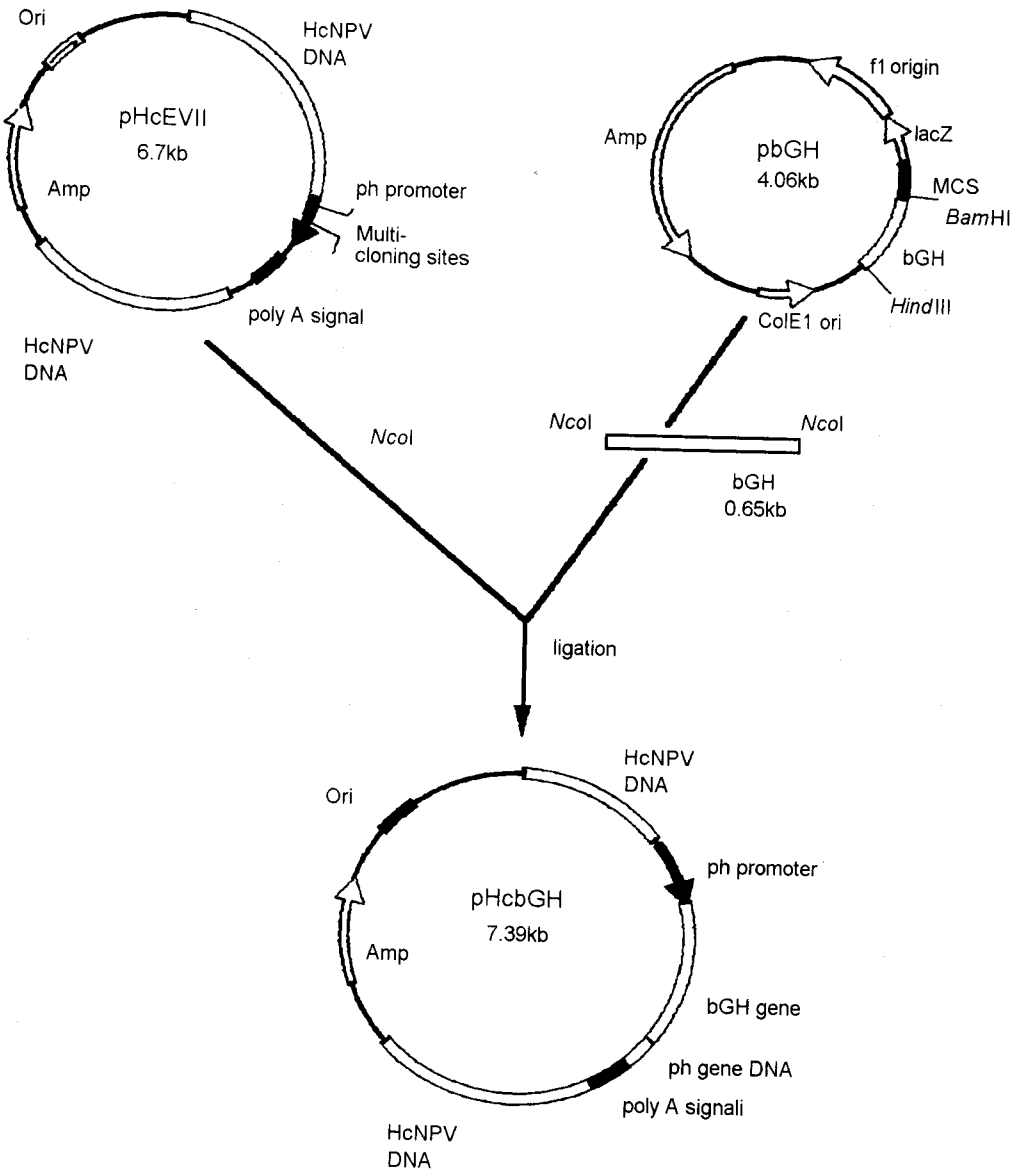

Fig. 3. Construction of pHcbGH recombinant plasmid. The bGH gene in the pbGH plasmid was transferred into the *Nco*I site of pHcEVII vector.

and hybridized with the probe DNA by the procedure of Southern [26]. DNAs were electrophorized on 0.8% agarose gel at 4℃ for 15h at 30 V/cm, denatured, neutralized, transferred on a Hybond-N^+ filter and then dried at room temperature for 5 minutes. The hybond-N^+ filter was hybridized to probe cDNA, exposed autoradiographically to hyperfilm ECL. The film was developed following the procedures suggested by Kodak.

Radioimmunoassay

Radioimmunoassay (RIA) was carried out as described by Teale [28] using RIA kit (^{125}I-HGH kit) (Radioassay Systems Laboratory, Inc. Carson, California) with slight modifications. First $2x10^7$ cells growing exponentially in suspension culture were seeded in tissue cul-

ture dishes (100x15 mm) and incubated for 24 h at 27℃ for attachment and growth, and then the cells were infected with the HcbGH-4-1 recombinant virus at a multiplicity of infection (m.o.i.) of 5 pfu per cell. After 1 h of adsorption at room temperature, the monolayers were washed with TNM-FH-T medium, and the cells were incubated with 5 ml of the medium for 5 days at 27℃. After incubation, cells were harvested, washed twice with TBS (2M tris, 4M NaCl, pH 7.5), 200l μl of 0.2% Triton X-100 was added to the cells and inverted thoroughly. Standing for 60 min on ice, cells were lysed by ultrasonic generator (Nissei, Japan). Supernatant was transferred to fresh tube and this sample was used for radioimmunoassay protein analysis using anti-human growth hormone.

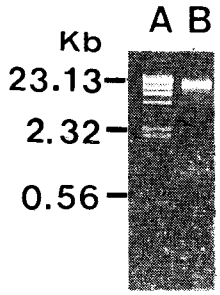

Fig. 4. Restriction pattern of pHcbGH recombinant DNA. Lanes A: λ DNA digested with *Hind*III enzyme and B, pHcbGH DNA digested with *Noc*I enzyme.

RESULTS AND DISCUSSION

Sequencing and cloning of bovine growth hormone

Previously the construction of a baculovirus expression and transfer vector, pHcEVII for eukaryotic cell using *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) was reported [20]. For utilization of the vector this investigation was undertaken and bovine growth hormone gene (bGH) [8] was used as a foreign gene to

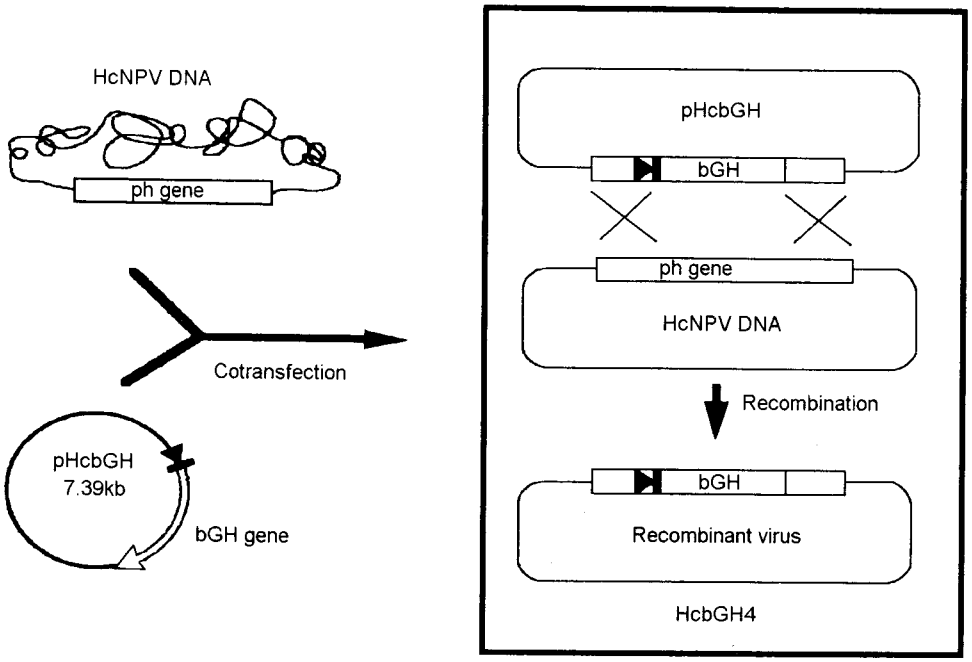

Fig. 5. Construction scheme of recombinant virus HcbGH-4-1 by cotransfection with HcNPV DNA and pHcbGH plasmid. *In vivo* homologous recombination will be occurred in the regions of polyhedrin gene in the both DNAs.

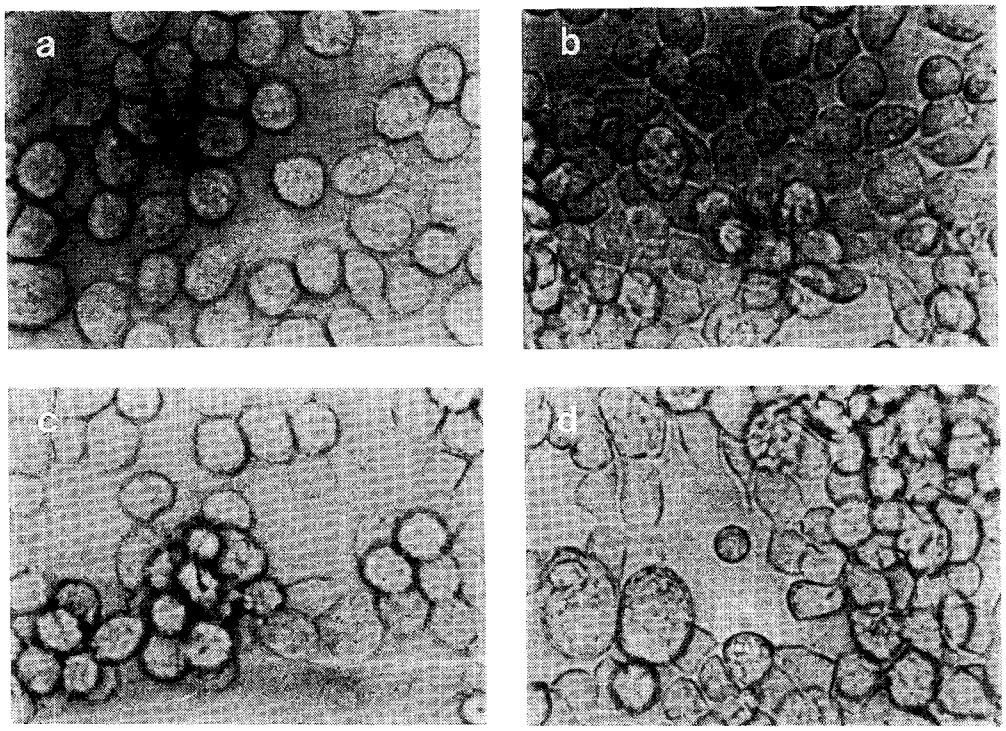

Fig. 6. *S. frugiperda* cells infected with recombinant virus, HcbGH-4-1. Cells infected with the recombinant virus did not form polyhedra in the cells and were hypertrophied (x 200). a: normal cell; b, cells infected at 24 h p.i.; c, cells infected at 48 h p.i.; and d, cell at 48 h p.i.

be expressed in the insect cell. Therefore, nucleotide sequences of the bGH gene and both its ends in the pbGH plasmid [8] was determined by the procedures described by Sanger et al., [22], and were shown in Fig. 2. This analysis revealed that the entire DNA fragment was 855 bp long and contained 654 bp (217 amino acid residues and one stop codon) open reading frame of the bGH gene, and the 56 bp 5'-flaking sequence upstream from the ATG translation initiation codon, and 145 bp 3'-untranslated regions. In the 3'-untranslational region there were two pallindromic regions (5'-TCCCCTCCCCCG-3', 5'-ACCCTGGAAGGT-GCCA-3'), the sequence AATAAA and polyA tailing regions (Fig. 2). This results were similar to the findings of Miller et al., [18]. This hexanucleotide is found in the 3' untranslated regions of most eukaryotic genes and has been shown to function in the poly adenylation of mRNA molecules.

*Nco*I sites were inserted before the translation start site (ATG) and behind the translation termination site (TAG) of the bGH gene in the pbGH clone for insertion of the bGH gene into *Nco*I site of the pHcEVII vector. The amplified bGH gene with the primers was approximately 0.654 kb (Fig. 3). This DNA was ligated into the *Nco*I site of the pHcEVII expression vector, which was named pHcbGH recombinant plasmid (Fig. 3). This plasmid DNA was redigested with *Nco*I enzyme for confirmation of its insertion (Fig. 4). Two bands of 6.74 kb and 0.65 kb appeared by *Nco*I digestion (Fig. 4B).

Construction of recombinant virus, HcbGH-4-1

The pHcbGH transfer vector DNA containing the bGH gene and the wild type HcNPV

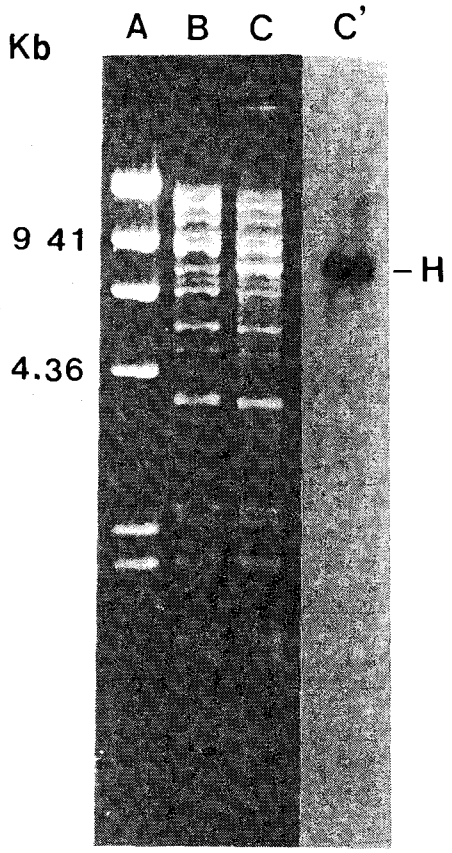

Fig. 7. Southern hybridization of HRP-labeled bGH gene cDNA to HcbGH-4-1 virus *Eco*RI fragment DNA. Lanes A: λ DNA digested with *Hind*III enzyme; B, HcNPV DNA digested with *Eco*RI enzyme; C, HcbGH-4-1 virus DNA digested with *Eco*RI and C', Southern hybridization of the lanes B and C with probe DNA. H indicates the hybrid bGH gene DNA.

DNA were cotransfected into *S. frugiperda* cells by the protocol described in Materials and Methods (Fig. 5). After cotransfection, recombination events that transfer the bGH gene from the transfer vector to the polyhedrin gene site of the HcNPV genome DNA were accomplished. The pHcbGH DNA and the HcNPV DNA were successfully cotransfected into *S. frugiperda* cells and multiplied.

Eight plaque clones of recombinant virus were isolated (Table 1). Individual clones were named HcbGH-1 to 8 on the basis of their selected order. The sizes of the plaques were

Table 1. Recombinant virus clones isolated

Plaque clones	Plaque size (mm)
HcbGH - 1	2.0
HcbGH - 2	0.5
HcbGH - 3	1.2
HcbGH - 4	3.0
HcbGH - 5	2.5
HcbGH - 6	2.1
HcbGH - 7	1.5
HcbGH - 8	1.8

Table 2. Radioimmunoassay of the bovine growth hormone produced in *S. frugiperda* cells infected with the recombinant virus HcbGH-4-1

Samples	Average amount (ng/ml) of bGH
LB broth (control)	<0.1
E. coli JM83/pHcbGH	<0.1
S. frugiperda cells	0.47
S. frugiperda cells/ infected with HcNPV	0.51
S. frugiperda cells/ infected with HcbGH	5.50

0.5 to 3.0 mm (Table 1). The plaques did not form polyhedral inclusion bodies in the cells. HcbGH-4 clone showed largest size of plaque. So, the clone was used for further studies. The plaques were plaque-purified twice, and then the large size plaque was named HcbGH-4-1, which was used in this study. The titer of the recombinant virus in the first infected supernatant was about 2.0×10^5 plaque forming units (pfu) per ml at 7 days postinfection (p.i), and the culture medium was used for the next infection inoculum. When the recombinant virus was infected to 3×10^7 cells with m.o.i. of 2, after 5 days p.i. the titer was 3×10^8 pfu per ml. These results indicate that the multiplication type of the recombinant HcbGH-4-1 was similar to that of the wild type HcNPV.

The recombinant virus, HcbGH-4-1 was synchronously infected to *S. frugiperda* cells, and then the morphogenesis of the cells was observed with phase contrast microscopy (Fig. 6).

From 2 to 5 days p.i., the nuclei of the cells were swollen and hypertrophied to the cell membranes and polyhedral inclusion bodies were not formed in the whole infected cells.

Confirmation of the bGH gene in the HcbGH-4-1 virus

The insertion of the bGH gene fragment in the recombinant virus, HcbGH-4-1 was confirmed by Southern blot. The purified recombinant viral DNAs were digested with the *EcoR*I enzyme and hybridized with bGH gene cDNA probe (Fig. 7). By the digestion with *EcoR*I enzyme the recombinant virus genome DNA was cleaved in twenty-four DNA fragments which were the same in numbers as the wild type HcNPV DNA pattern (Fig. 7), however the *EcoR*I-H fragment thicker than that of the wild type HcNPV fragment (Fig. 7C). The probe DNA was hybridized to *EcoR*I-H fragment of HcbGH-4-1 recombinant DNA with high density (Fig. 7C'). This result indicates that the gene was inserted into the recombinant virus, HcbGH-4-1.

Detection of the bGH in *S. frugiperda* cells infected with the HcbGH-4-1 virus

The production of the bovine growth hormone in the *S. frugiperda* cells infected with the HcbGH-4-1 recombinant virus was measured by radioimmunoassay [28]. The production of the bovine growth hormone in *E. coli* and *S. frugiperda* cells containing the recombinant virus are shown in Table 2. *E. coli* JM83 which contains pHcGH transfer vector did not produce bGH protein. The amount of bGH protein expressed in *S. frugiperda* cells infected with HcbGH-4-1 recombinant virus was 5.5 ng per ml (10^6 cells).

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