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Recombination and Expression of VP1 Gene of Infectious Pancreatic Necrosis Virus DRT Strain in a Baculovirus, *Hyphantria cunea* Nuclear Polyhedrosis Virus

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=국문초록=

전염성 췌장괴저바이러스 DRT Strain VP1유전자의 Baculovirus *Hyphantria cunea* Nuclear Polyhedrosis Virus에 재조합과 발현

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전염성 췌장괴저바이러스 (Infectious pancreatic necrosis virus) DRT 주의 VP1유전자를 대장균 발현 운반체와 Baculovirus에 삽입하여 대장균과 진핵세포에서 VP1단백질의 발현을 연구하였다. 재조합체 pMal-pol 클론 [7] 에서 2.7 Kb 단편인 VP1 유전자를 제한효소 *Xba*I으로 절단하여 Baculovirus 운반체인 pBacPAK9에 클로닝하여 pBacVP1이라 명명하였다. 이 pBacVP1에 클로닝된 VP1유전자를 제한효소 *Sac*I과 *Pst*I으로 절단하여 대장균 발현 운반체인 pQE-30에 클로닝하여 pQEVP1이라 명명하였다. 또한 VP1 단백질의 C-말단에 6개의 히스티딘 6×His이 붙어 있는 단백질을 만들기 위하여, pQEVP1 클론의 His부위를 *Eco*RI으로 절단하고, 또한 pBacVP1을 *Eco*RI으로 절단하여 생긴 부위에 His-*Eco*RI DNA 단편을 교체시켜 재클로닝하여 pBacHis-VP1을 만들었다. pBacHis-VP1 DNA와 *Bsu*36I로 처리된 *LacZ-Hyphantria cunea* nuclear polyhedrosis virus (*LacZ*-HcNPV)를 함께 lipofectin을 이용하여 곤충세포 (*Spodoptera frugiperda* cell)에 동시감염을 시켜서 재조합 바이러스를 선발하여, VP1-HcNPV-1이라 명명하였다.

pQEVP1 클론은 6개의 히스티딘 단편이 부착된 VP1단백질을 Ni-NTA resin크로마토그래피법으로 정제하여 SDS-PAGE와 Western blot으로 확인하였고, 단백질의 활성과 구조에 영향을 주지 않는 6개의 히스티딘 단편 (6 × His)이 부착된 94 kDa의 VP1 단백질을 정제할 수 있었다. 또한 재조합 바이러스에 감염된 곤충세포에서 VP1 단백질이 발현된 것을 전기영동과 Western blot으로 검색을 한 결과 95 kDa VP1 단백질이 발현이 되었음을 확인하였다.

Key Words: Infectious pancreatic necrosis virus, VP1 gene and protein RNA dependent RNA polymerase, Baculovirus

INTRODUCTION

The RNA genome B segment (approximately 2.8 Kb) of infectious pancreatic necrosis virus (IPNV) contains single open reading frame and encodes VP1 protein VPg [1] which is associated with RNA-dependent RNA polymerase [2,3]. The VP1 protein is present in two forms; as a free polypeptide and as a genome-linked protein (VPg). In the latter form it is

linked to the 5' end of both genome segments by a serine-5'-GMP phosphodiester bond [2]. The VP1 may also contain guanylyl and methyl transferase activities [4].

The nucleotide sequences of the genome RNA segments A and B of IPNV-DRT strain were 3,155 bp and 2,783 bp respectively [5,6]. In the previous report the RNA genome B segment was synthesized in three fragments by reverse transcriptase (RT)-polymerase chain reaction and combined together to construct full length of cDNA of the VP1 gene, and then it was cloned and expressed in *E. coli* system [7]. The VP1 gene was expressed in the cell and produced 94 kDa protein.

Baculovirus vector for eukaryotic expression are developed using the strong promoters of *Autographa californica* nuclear polyhedrosis virus [8] and *Hyphantria* cunea nuclear polyhedrosis virus polyhedrin genes [9]. Many researchers are using eukaryotic expression vectors, rather than prokaryotic or lower eukaryotic vectors, to optimize the likelihood that the product will be biologically active. Many factors may affect the biological activity of eukaryotic protein, including post-translational modifications and the tertiary structure (e.g. disulfide bond formation) or quaternary structure (e.g. oligomerization or complex formation) [8]. Moreover, insect cells carry out most of the post-translational processing events that occur in mammalian cells so that eukaryotic proteins produced by a baculovirus expression system are usually similar to the authentic protein in their biological activity, structure and antigenicity [10]. The first perfect protein of human β-interferon was produced using baculovirus expression system [11].

To compare the expressions of the VP1 gene of IPNV in prokaryotic and eukaryotic cells, the VP1 gene of IPNV was expressed in *Spodoptera frugiperda* cell using a Baculolvirus expression system. In this article we report the cloning and the expression of the cDNA of the VP1 gene of IPNV-DRT strain. We have cloned the full-length cDNA of RNA-dependent RNA polymerase (VP1) gene into Baculovirus transfer vectors and recombinated the gene with *LacZ-Hyphantria cunea* nuclear polyhedrosis virus. Then recombinant baculovirus was selected and expressed in *Spodoptera frugiperda* cells.

MATERIALS AND METHODS

1. Virus and Cell

LacZ-Hyphantria cunea nuclear polyhedrosis virus (*Lac*Z-HcNPV) was obtained in this lab and was used for construction of recombinant virus. The virus inoculum used was extracellular nonoccluded virion (NOV) derived from cell culture media. The *Spodoptera frugiperda* cell line (IPLB-SF-21) [12] was obtained from Dr L. K. Miller (University of Georgia and used for propagation and plaque assay of NOV [13]. *S. frugiperda* cell was routinely cultured at 28℃ in a TC-100 medium (Gibco BRL, Grand Island, NY) supplemented with 0.26% tryptose broth (Difco, Detroit, mi) and 10% fetal bovine serum (Gibco). The media were contained 50 μg of gentamycin per cell (Gibco).

2. Bacterial strains, plasmids, transfer vector and oligonucleotides

Escherichia coli XL1-Blue (Stratagene, La Jolla, CA) and pMal-pol clone [7] containing the VP1 gene were stored at Research Institute for Genetic Engineering, Konkuk University. *Escherichia coli* M15 and pQE-30 expression vector were purchased from QIAGEN, (Chatsonworth, CA.) *E. coli* M15 was used as expression host. pBacPAK9 transfer vector was purchased from Clontech Co. (PaloAlto, CA) Primer Hc-1 (5'-TGATAACCATCTCGC-3') was used for sequencing and purchased from Bioneer Co. (Daejon, Korea).

3. Preparations of VP1 protein antibodies

IPNV polyantibodies was obtained from Dr. Peter, Dobos (Department of Microbiology, University of Guelph, Canada) and mouse monoclonal^{MRGS}. His antibody was purchased from QIAGEN Ins.

Also antibody against MBP-VP1 fusion protein was also prepared. The 0.5 μg fusion protein, MBP-VP1 produced by pMal-pol clone [7] and equal volume of complete Freund's adjuvant (Gibco) were

mixed and injected into a peritoneal cavity of a mouse. After 3 weeks, the 0.5 μg fusion protein and an equal volume of incomplete Freund's adjuvant (Gibco) was also mixed and injected into the peritoneal cavity. To obtain an antiserum after 10-14 days, it was collected from the heart and incubated at 4℃ for 18 h. It was centrifuged at 2,700 ×g for 10 min. Then the supernatant was stocked as antiserum at -20℃.

4. Reagents

All restriction endonucleases, T4 DNA ligase and calf intestinal alkaline phosphatase (CIAP) were purchased from New Englad Biolabs (NEB), Maryland, and Boehringer Mannheim Biochemica (BM), Mannheim. ECL gene detection system and [α-^{35}S] dATP were obtained from Amersham (Lfe Science, Cleveland, Ohio) sequenase version 2.0 DNA sequencing kit was purchased United State Biochemical (USB, Cleveland). Ni-NTA resin was purchased from QIAGEN Ins. and amylose resin was purchased from USB. Another reagents were purchased from Difco lab, Gibco, Sigma Chemical (St. Louis, MO) and Promega (Madison, WI).

5. Preparations of NOVs of *Lac*Z-HcNPV

The *S. frugiperda* cell monolayers in 75 cm^2 tissue culture flasks were inoculated with NOVs of *Lac*Z-HcNPV at a multiplicity of infection (m.o.i.) of 2 pfu per cell and were incubated at 28℃. When a complete cytopathic effect was observed in infected cells by 5 days postinfection (p.i.), they were scraped into the media with policeman. Cells and virions fluid were centrifuged at 13,000 ×g for 30 mins at 4℃. The cell pellets were removed. 3.0 ml of sucrose cushion solution (25% sucrose in 5 mM NaCl, 10 mM EDTA) was loaded in each of six 38 ml polyallomer ultracentrifuge tubes (Beckman, Sormerset, NJ, SW28) and overlaid carefully with 33 ml supernatants containing NOVs. The virus stock was centrifuged at 100,000 ×g in an SW28 rotor of Beckman Instruments (Sormerset, NJ) for 2 h at 4℃ and discarded the supernatant and carefully removed traces of sucrose. The NOV pellets were resuspended in a total of 1.0 ml TE buffer (pH 8.0).

6. Viral DNA purification

The concentrated NOV pellets in 1.0 ml TE (pH 8.0) buffer were treated with 100 μl of 10% SDS, 0.01 g of *N*-laurylsarcosinate and proteinase K (200 μg/ml) and incubated for 3 h at 37℃. When the solution was clear, the DNAs were purified by sequential phenol, phenol-chloroform-isoamylalcohol (25 : 24 : 1), and chloroform-isoamylalcohol (24 : 1) extraction.

Extracted DNA solution was dialyzed at 4℃ against 500 volumes of TE buffer with two changes for at least 4 h each time. After the dialysis, the DNA solution was adjusted to be a final concentration of 0.3 M sodium acetate and centrifuged at 30,000 ×g. DNAs in the supernatants were precipitated by 2.5 volumes of 95% ethanol at -20℃ for overnight. DNA precipitates were pelleted by microcentrifugation at 17,000 ×g (Tomy Seiko Co., Tokyo) for 15 min at 4℃. The DNA pellets were washed with 70% ethanol, dried, redissolved in 5 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA buffer and stored at 4℃.

7. Purification of plasmid DNAs

E.coli containing recombinant plasmids was cultured in LB broth (Difco) at 37℃ for 18 h and then the plasmid DNA was isolated by alkaline method procedure and purified by the Birnboim and Doly [14] procedure.

8. Restriction endonuclease digestion and agarose electrophoresis

The DNA was digested with restriction enonucleases as recommended by the suppliers. The fragments were analyzed in 1.0% agarose gels using tris-borate buffer system. Details of gel electrophoresis and visualization of the DNA fragments have been described [15]. The molecular size of each DNA fragment was determined by comparing its mobility with standard DNA fragments known sizes.

9. SDS-polyacrylamide gel electrophoresis

Protein samples were analyzed by 7.5% SDS-polyacrylamide gel as described by Laemmli [16].

10. Construction of baculovirus transfer vector pBacVP1

The construction scheme of pBacVP1 clone is illustrated in Fig. 1. pMal-pol clone [7] containing the VP1 gene (2.7 kb) was digested with XbaI enzyme and then the VP1 gene DNA fragment was electrophoresed in 1% Seakem agarose gel. The gene DNA fragment vizualized on the agarose gel was cut and recovered by Gene clean® III kit (BIO 101, Inc. La Jolla, CA). At the same time pBacPAK9 transfer vector was digested with *Xba*I enzyme, and then treated with 0.01 unit of CIAP for 60 min at 37℃, extracted with phenol, precipitated with ethanol, and suspended in distilled water at 1.0 mg/ml to prevent self-ligation. The VP1 gene was ligated into *Xba*I site of the pBacPAK9 transfer vector by T4 DNA ligase which was named pBacVP1 recombinant plasmid. The ligation mixture was mixed with $CaCl_2$-treated *E. coli* XL-1 Blue, kept on ice for 60 min, and then heat-shocked at 42℃ for 90 sec. After the aliquot was placed on ice again for 5 min, it was directly smeared on LB agar medium with ampicillin (50 μg/ml) for selection of transformed colonies. This plate was incubated at 37℃ for 16 h. The colonies were isolated and identified. To confirm insertion of VP1 gene, DNA sequencing was partially performed.

11. Construction of pQEVP1 clone

The construction scheme of pQEVP1 clone is illustrated in Fig. 2. The pBacVP1 (8.2 Kb) subclone containing the VP1 gene was digested with *Pst*I and *Sac*I enzymes, and then the VP1 gene DNA fragment was run and eluted by Gene clean III kit. At the same time pQE-30 expression vector containing 6xHis affinity tag coding sequence was digested with PstI and SacI enzymes, and then treated CIAP to prevent self-ligation. The VP1 gene was ligated into the enzyme sites of the pQE-30 vector by T4 DNA ligase. The ligation mixture was transformed in *E. coli* XL-1 Blue with the method mentioned in previous experimient. The transformed *E. coli* cells were incubated at 37℃ for 16 h. The colonies were isolated and identified. It was named pQEVP1 recombinant.

12. Construction of transfer vector pBacHis-VP1

The construction scheme of pBacHis-VP1 clone is illustrated in Fig. 3. pBacVP1 (8.2 Kb) subclone containing the VP1 gene was digested out with *Eco*RI enzyme, and then DNA fragments were run and eluted by Geneclean® III kit. At the same time the pQEVP1 (6.2 Kb) clone was digested with *Eco*RI enzyme and then the *Eco*RI fragment was treated with CIAP to prevent self-ligation. The *Eco*RI fragment containing 6×His-tagged VP1 gene was ligated into the *Eco*RI site of the pBacVP1 recombinant by T4 DNA ligase. The ligation mixture was transformed in *E. coli* XL-1 Blue. The transformed *E. coli* cells were incubated at 37℃ for 16 h. The colonies were isolated and identified. It was named pBacHis-VP1 recombinant. The VP1 gene was inserted just before the polyhedrin promoter. For confirmation of the *Eco*RI fragment of the 6xhis-tagged VP1 gene and the part of pBacPAK9, multicloning sites of the pQE-30 region and the upstream region of the VP1 gene were sequenced.

13. Construction of a recombinant baculovirus with VP1 gene

The construction scheme of a recombinant baculovirus is illustrated in Fig. 4. *S. frugiperda* cells were cotransfected with linear *Lac*Z-HcNPV DNA digested with *Bsu*361 enzyme and pBacHis-VP1 subclone containing the 6×His-tagged VP1 gene DNA using lipofectin technique [17]. *In vivo* homologous recombinations between the recombinant plasmid DNAs and the viral DNAs were occurred and in the process the target gene was transferred into the viral genome (Fig. 4).

Exponentially growing *S. frugiperda* cells were seeded in two 25 mm dishes with 3×10^6 cells and incubated at 28℃ for 2∼24 h to allow the cells to attach. The old medium was removed from the cultures and washed twice with new 2 ml of basal TC-100 (no serum, no antibiotics). The cells were incubated at room temperature for 10∼30 min while the transfection reagent-DNA mixture is prepared as

described in the following steps. The following additions were prepared in two sterile polystyrene tubes: one for cotransfection mixture solution (40 μl H_2O, 5 μl of the 100 ng/μl pBacHis-VP1 plasmid DNA solution, and 5 μl *Lac*Z-HcNPV DNA digested with *Bsu*361), and another for the control mixture solution (40 μl H_2O, 5 μl of the 100 ng/μl pBacHis-VP1 plasmid DNA solution and without the *LacZ*-HcNPV DNA).

50 μl of the transfection reagent (5 μl lipofectin plus 45 μl distilled water) was added to the tubes, mixed gently, incubated at room temperature for 15 min to allow the transfection reagent to form complexes with the DNA. Meanwhile, the medium was removed from the cell monolayers and then the cells were washed twice with 2 ml of basal TC-100 medium. 2.5 ml of the basal TC-100 medium (no serum, no antibiotics) containing gentamycin was added to the cell monolayers. The lipofectin-DNA complexes were added dropwise to the medium while the dishes were gently swirled. After incubation at 28℃ for 5 h, 2.5 ml of the TC-100 medium containing 10% fetal bovine serum and antibiotics were added to each dish and then incubated at 28℃ for 60~72 h. After addition of the lipofectin-DNA mixture to the cells, the medium which contains recombinant viruses produced by the transfected cells was transferred to a sterile container and stored at 4℃. X-gal-plaque assay was carried out with the cotransfected supernatants at dilutions of 10^{-1}, 10^{-2}, and 10^{-3} to select recombinant viruses by white plaque color.

14. Plaque assay

Plaque assays were carried out as described by Lee and Miller [13], with the following slight modifications to determine the titration and to detect recombinant viruses. *S. frugiperda* cells (2×10^6 cells per 1.0 ml of complete TC-100 medium) were seeded into a 60×10 mm tissue culture petri dish and incubated at 28℃ for 24 h. Ten-fold dilutions of the recombinant virus were prepared in PBS (pH 6.2). The medium was removed from the cell culture, which was inoculated with 0.1 ml of the virus inoculum for 60 min with gentle shaking at the intervals of 15 min. After 60 min, the liquid on the cells was washed out with PBS buffer. The cell monolayers were covered with 5 ml of the overlay medium (0.8% low melting temperature agarose, 20 μl/ml of X-gal (5-bromo-4-3-indolyl-β-D-galactopyrnoside, and 20 μg/ml IPTG (isoprophyl-thio-β-D-galactoside in the complete TC-100 medium). The cultures were incubated at 28℃ for 4 days, and then at the interval of every 12 h, white plaques were detected by inverted microscopy to confirm whether they were produced polyhedra or not. Then the white plaques were isolated and titered for further plaque-purifications.

15. Analysis of VP1 protein produced in *E. coli* cells by pQEVP1 clone

1) Checking for the VP1 protein location in *E. coli* cell

The *E. coli*/pQEVP1 clone was inoculated into 20 ml of LB-broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. To find the best temperature and IPTG level, the pQEVP1 clone was grown at 23℃, 30℃ and 37℃. Until the optical density at 600 was reached to 0.5, the pQEVP1 clone was continuously cultured. One ml sample was taken before induction (uninduced sample). IPTG was added to the culture at the final concentration of 2 mM and then continuously cultured for 4 h (induced sample). After the culture was divided into two aliqots, the cells were harvested at 4,000 ×g for 10 min. To check for cytosolic locallization, one pellet was resuspended in 5 ml of sonication buffer (50 mM Na-phosphate pH 7.8, 300 mM NaCl) and freezed at -70℃ (sample A). Sample A was thawed in cold water and sonicated briefly to lyse the cells. The sample A was centrifuged at 10,000 ×g for 20 min and decanted the supernatant (crude extract 1, soluble protein). The pellet was resuspended in 5 ml sonication buffer and then sonicated. This is a suspension of the insoluble matter (crude extract 2, insoluble protein). To check for periplasmic localization, the other pellet was resuspended in 10 ml of the solution (30 mM Tris-HCl, 20% sucrose, pH 8.0), which was named sample B. Sample B was added 1.0 mM EDTA was

added to sample B and then it was incubated at room temperature for 5～10 min with shaking. After sample B was centrifuged at 8,000 ×g at 4℃ for 10 min, all the supernatant was removed and the pellet was resuspended in 10 ml ice cold 5 mM $MgSO_4$. The sample B was shaked or stirred in an ice water bath and centrifuged at 8000 ×g at 4℃ for 10 min. The supernatant is the osmotic shock fluid (extract 3, periplasmic extract). The samples were analyzed by 7.5% SDS-polyacrylamide gel [16].

2) Purification of VP1 protein by Ni-NTA resin column

The 6×His-tagged VP1 protein which was produced by *E. coli* M15/ pQE-VP1 clone was purified on Ni-NTA chromatography (QIAGEN Inc, Valencia, CA.). Ten ml of the overnight culture of *E. coli* M15/pQE-VP1 clone grown at 30℃ was inoculated in 500 ml of the LB broth (10 g Bacto trypton, 5 g yeast extract, 5 g NaCl) containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. After grown at 30℃ with vigorous shaking until the optical density at 600 was reached to 0.7～0.9, IPTG was added to be a final concentration of 1～2 mM and continued to grow the culture at 30℃ for 5 h.

The cell was harvested by centrifugation at 4,000 ×g for 20 min and stored at -70℃ overnight. The pellet was thawed for 15 min and resuspended in 8 ml of buffer A (6 M GuHCl, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 8.0) for solubilization of the protein in inclusion bodies. The mixture was stirred for 1.0 h at room temperature and harvested at 10,000 ×g for 15 min at 4℃.

The collected supernatant was mixed with 8 ml of a 50% slurry of Ni-NTA resin, stired at room temperature for 45 min, and then loaded resin carefully into a 1.6 cm diameter column. The column was washed 10 column volumes of buffer A and 5 column volumes of buffer B (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 8.0). Next, the column was washed with buffer C (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl, pH 6.3) until the optical density at 600 was reached to 0.01. The 6×His-tagged protein was eluted with 20 ml of buffer D (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris, pH 5.9) and buffer E (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris, pH 4.5). Each elution was collected by 3 ml fractions. The purified protein was further characterized by 7.5% SDS-polyacrylamide gel elecrophoresis [16].

16. Analysis of VP1 protein in *S. fruigperda* cell infected by recombinant virus with Western blot

S. frugiperda cells infected with a recombinant virus at a m. o. i. of 5 pfu per cell were harvested by centrifugation at 3 days p.i. Samples of infected cells and uninfected cells were solubilized in electrophoretic sample buffer (60 mM Tris-HCl buffer pH 6.8 containing 2% (w/w) SDS, 25% (v/v) glycerol, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue) by heating at 100℃ for 4 to 5 min. After SDS-PAGE, the gel was either stained with Coomassie blue R-250 (Sigma Chemicals, St. Louis, MO) for 20 min or used for Western blot [18]. The gels was stained with Coomassie blue to confirm complete transfer of the proteins. The nitrocellulose filters (Amersham) were blocked with 3% BSA in tris buffered saline (TBS; 10 mM Trisma base, 150 mM NaCl, pH 7.5) for 60 min at 37℃ and washed three times with the TBS. It was incubated at 37℃ with a 1:1,000 dilution of rabbit anti-MBP-VP1 serum and washed three times with the TBS. Then it was incubated for 60 min at 37℃ with 1 : 1,000 dilution of horse radish peroxidase conjugated anti-goat mouse IgG and IgM (Jackson Immuno-Research Lab). After washing twice with the TBS, it was developed with development solution (30 mg chloronapthol per 1.0 ml methanol, 10 ml methanol, 50 ml TBS, 30 μl H_2O_2).

17. DNA sequence analysis

DNA sequence of the VP1 gene (2.7 Kb) in the pBacVP1 and the pBacHis-VP1 clones were carried out as described by the dideoxy chain termination method [19], with the following slight modifications. Hc-1 primer, 7-deaza-dGTP sequencing kit with sequenase version 2.0, and T7 DNA polymerase (United States Biochemica, Cleveland, Ohio) were used. The double-stranded DNA template (4～8 μg/μl) was

added to 2 μl of primer (100 pmol) and denatured in 0.2 N NaOH, 0.2 mM EDTA at 37℃ for 30 min. It was precipitated with 50 μl of absolute ethanol in the presence of 2 μl (0.1 volume) of 5 M ammonium acetate (pH 4.6) at -70℃ for 30 min, and then centrifuged at 4,000 ×g for 15 min. After washing the pelleted DNA with 70% ethanol, it was dissolved in 8 μl of H_2O and added to 2 μl of 5× sequencing reaction buffer. The mixture was warmed at 65℃ for 2 min, then allow the temperature to cool slowly to room temperature over a period of about 30 min. Once the temperature is below 30℃, annealing is complete. The annealed template-primer was added by 1 μl of 0.1 M dithioreitol, 2 μl of diluted 5× labelling mixture, 0.5 μl of [α-^{35}S] dATP (10 ci/μl, 10 mM) and 2 μl of diluted sequenase on ice. The reaction mixture was incubated at room temperature for 5 min.

For the termination of reaction, it was prepared that 4 eppendorf tubes were filled with 2.5 μl of the 7-deaza-dGTP, 7-deaza-dATP, 7-deaza-dTTP and 7-deaza-dCTP respectively. When the reaction mixture incubation was completed, removed 3.5 μl and transferred it to each of 4 tubes, and then incubated for 5 min in 37℃ water bath. Finally, it was added 4 μl of stop solution to each of 4 tubes for the termination of the reaction. These samples labelled with ^{35}S were stored at -20℃ until they were loaded in sequencing gel. Six percentage SDS-PAGE gel [16] was prepared 2~20 h prior to use, and pre-run for 15~60 min. When the gel is ready for loading, the samples were heated at 80℃ for 2 min and immediately loaded 2.5 μl in each lane on 6% denaturing polyacrylamide gel. The gel was run at 1,500 Volts for 2~6 h.

The ^{35}S-gel was dried and exposed onto Kodak Biomax MR film (Kodak, Rochester, NY) at -70℃ overnight to 72 h.

18. RNA polymerase activity assay

RNA polymerase assay was carried out as described by method of Mertens *et al.* [20], with the following slight modification. *S. frugiperda* cells infected and uninfected with a recombinant virus at a m. o. i. of 5 were harvested at 4 days p.i. Samples of infected cells and uninfected cells were washed once in phosphate buffered saline (PBS) and resuspended in lysis buffer (50 mM tris-HCl, pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl, 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 20% glycerol). The cells were disrupted in this buffer using a sonicator (Biosonik BP-111, Braunwell Scientific, Rochester, NY). After sonication, the lysates were immediately frozen at -70℃ and stored until use. The lysates of virus particles released into the culture media were prepared in a similar method. Culture medium in which the cells were grown was filtered through 0.22 μm filter. The filtrate was then centrifuged at 100,000 ×g for 120 min with a SW28 rotor in a ultracentrifuge (Kontron Instruments, Zurich). The viral pellet was resuspended in lysis buffer and sonicated as above.

The standard reaction mixture was prepared by the method of Mertens *et al.* [20] and pre-tests showed that the lowest, non-limiting concentration of CTP that could be used in a reaction mixture for up to 5 h incubation, was 0.02 mM. The addition of α-^{32}P-CTP (Amersham) increased the total CTP concentration to 0.0225 mM per 0.1 ml assay. Assay components were prepared as stock solution (usually 10 × conc) in sterile glass-distilled water. The reaction mixture (15 μl of lysate was mixed with 15 μl of the standard reaction mixture.) was incubated at 28℃ in sterile 1.5 ml Eppendorf tubes. Assays were performed in duplicate and duplicate 5 μl samples were removed from both assays, spotted onto Whatman GF/C filter paper and washed with 10 ml of cold 5% trichloroacetic acid (TCA)/1.0% sodium pyrophosphate, followed by two washes with 10 ml of 95% ethanol. After drying, the TCA-insoluble radioactivity on the filters was determined by Somogyi and Dobos [21]. The filters were placed in scintillation vials (5.5×1.5 cm) with 3 ml of toluen-based scintillation fluid (Luma gel, Lumc systems Inc, Netherland) and counted in a liquid scintillation counter (1217 RackBeta, LKB- Productor, Bromma, Sweden).

RESULTS

1. Construction of recombinant baculovrius, VP1-HcNPV-1

The VP1 gene of infectious pancreatic necrosis virus DRT (IPNV) strain RNA genome segment B cDNA coding 94 kDa polypeptide was cloned into baculovirus transfer vector and then constructed a recombinant baculovirus.

The VP1 gene (2.7 kb) in the recombinant plasmid pMal-pol [7] was used. The VP1 gene in the clone was first transferred into the transfer vector, pBacPAK9, and then the resulting recombinant was named pBacVP1 clone (8.2 Kb) (Fig. 1). The VP1 gene was inserted behind the intact polyhedrin promoter of *Autographa californica* NPV in the vector plasmid. The insertion of the VP1 gene in the pBacVP1 clone was confirmed by restriction enzymes *Xba*I, *Sma*I, *Hind*III, *Eco*RI and *Bgl*II (Fig. 2) and by sequencing (Fig. 3). By *Xba*I the DNA was cleaved into two fragments 2.7 and 5.5 kb; by *Sma*I three fragments 0.6, 1.8, and 5.8 kb; by *Hind*III two fragments 2.9 and 5.3 kb; by *Eco*RI two fragments 2.4 and 5.8 kb, and by *Bgl*II two fragments 0.8 and 7.4 kb. The Fig. 3 is the sequences of the regions of the cloning site-*Xba*I site -start codon area, which indicate the gene is correctly inserted.

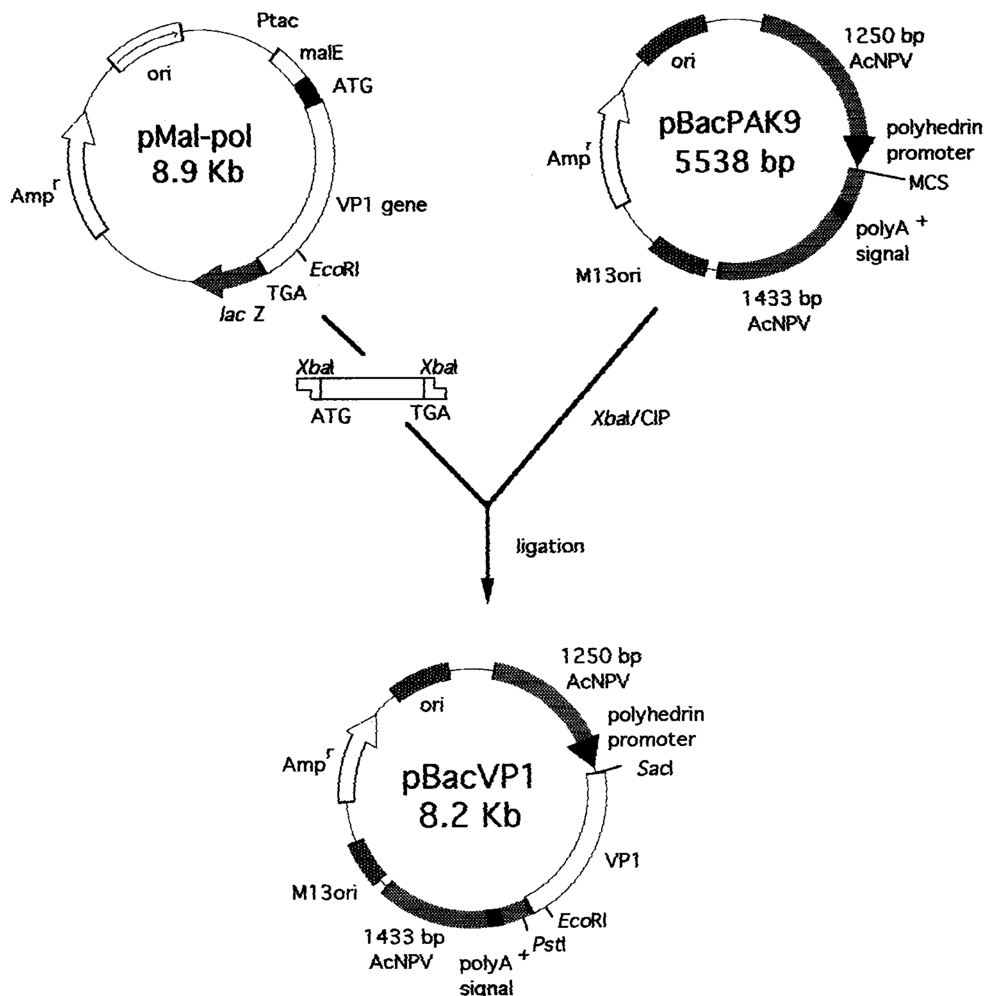


Fig. 1. Construction of Baculovirus transfer vector pBacVP1. The VP1 gene in the pMal-pol recombinant was subcloned into the pBacPAK9.

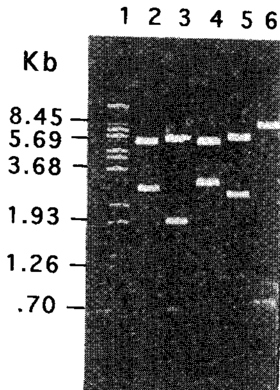
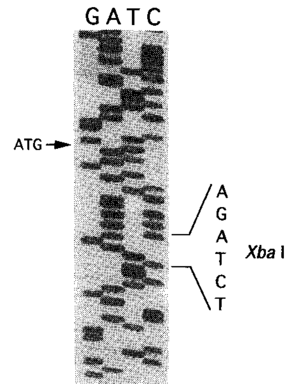


Fig. 2. Restriction fragment pattern of pBacVP1 DNA. pBacVP1 DNA was digested with restriction enzymes and electrophoresed in 1% agarose gel. Lane 1, λDNA digested with *Bst*EII. Lanes 2, pBacVP1 digested with *Xba*I; 3, with *Sma*I; 4, with *Hind*III; 5, with *Eco*RI; and 6. with *Bgl*II.



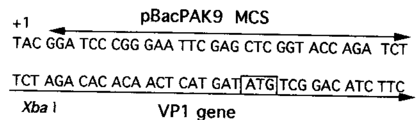


Fig. 3. DNA sequence of the cloning site-*Xba*I site-start codon area of pBacVP1 clone. This sequence was identified by dideoxy chain termination method with Hc-1 primer.

The VP1 gene in the pBacVP1 was transferred into the expression vector pQE-30, and the resulting recombinant clone was named pQEVP1 (6.16 Kb) (Fig. 4). The VP1 gene is inserted behind T5 phage promoter and the 6×His region of pQE-30 vector. Also the insertion of the VP1 gene in the pQEVP1 clone DNA was confirmed by restriction enzymes, *Xba*I, *Sac*I, *Pst*I, *Eco*RI, *Hind*III, *Nde*I, *Pvu*II, *Xho*I and *Sma*I (Fig. 5). By *Xba*I the clone DNA was cleaved into three fragments 1.0, - 2.7, by *Sac*I and *Pst*I two 2.7 and 3.4, by *Eco*RI two 2.5 and 3.6, by *Hind*III two 2.4 and 3.8, by *Nde*I/*Pst*I two 1.2 and 4.9, by *Pvu*II five 0.07 - 3.8, by *Xho*I four 0.2 - 3.2, and by *Sma*I two 0.6 and 5.6.

The 6×His-VP1 gene region in the clone pQEVP1 was transferred into the *Eco*RI site of transfer vector pBacVP1, and the resulting transfer vector was named pBacHis-VP1 (Fig. 6). In this recombinant transfer vector the VP1 gene was inserted behind polyhedrin promoter and the 6×His region. The pBacHis-VP1 was analyzed and confirmed by restriction enzymes, *Xba*I, *Bgl*II, *Pvu*II, *Eco*RI and *Sma*I (Fig. 7). By *Xba*I the clone DNA was dingested into two fragments 2.7 and 5.6, by *Bgl*II two 0.7 and 7.6, by *Pvu*II four 0.08 - 6.3, by *Eco*RI two 2.5 and 5.8, and by *Sma*I three 0.6 - 5.8. Right orientation and cloning in the regions of polyhedrin promoter-ATG-6×His-*Xba*I-starting region of the VP1 gene transcription were confirmed by sequencing (Fig. 8). These results confirm that the VP1 gene is correctly inserted and oriented in the vector.

Finally the VP1 gene in the pBacHis-VP1 clone DNA was recombinated into the *LacZ* gene site of the host virus *LacZ*-HcNPV on *S. frugiperda* cells by the lipofection-mediated method. Recombination was occurred between the homologous sequences flanking the *Bsu*361 cleaved regions of the linear *LacZ*-HcNPV and the both ends of polyhedrin gene in the vector (Fig. 9). The recombinant virus was selected and characterized by plaque assay containing X-gal. The white color plaques without the polyhedral inclusion bodies. were isolated as a recombinant virus. One selected recombinant virus was named VP1-HcNPV-1.

The normal *S. frugiperda* cells were not formed any inclusion bodies in its cell (Fig. 10A). The formation of the inclusion bodies in the cells infected with wild type *LacZ*-HcNPV was microscopically observed at 5 days p.i. (Fig. 10B). The plaque formed in the cells infected with the VP1-HcNPV-1 recombinant virus is illustrated in Fig. 10C, no formation of the polyhedral inclusion bodies. At 5 days p.i. the nuclei of the cells infected with the *LacZ*-HcNPV or the VP1-HcNPV-1 were swollen and the nuclear membranes were hypertrophied to

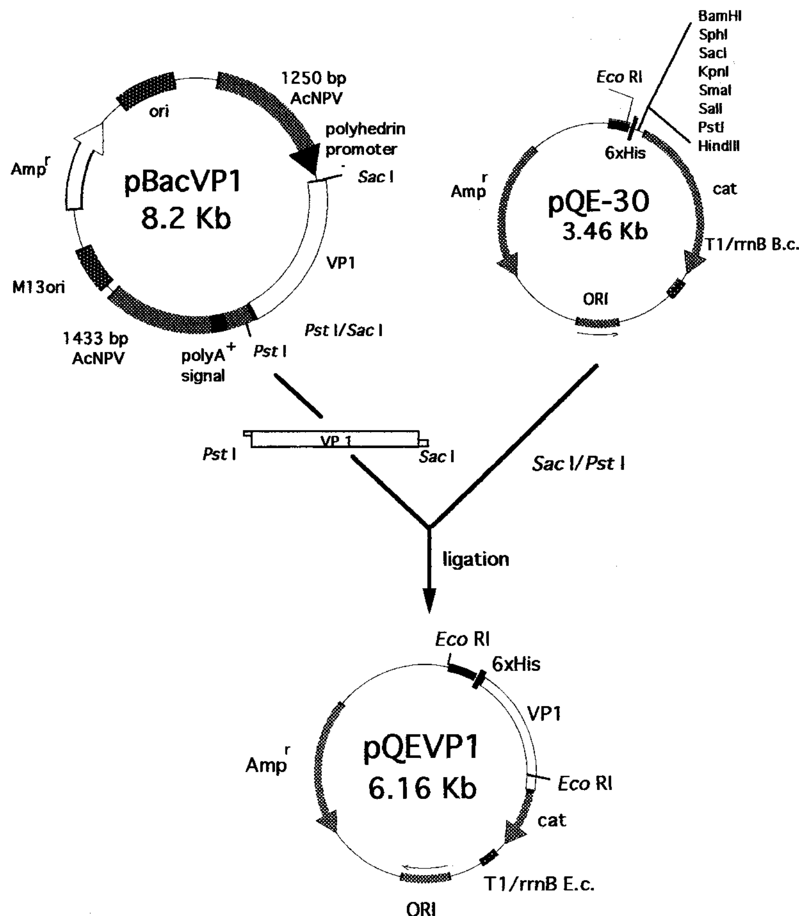


Fig. 4. Construction of pQEVP1 subclone. The VP1 gene sequence in the pBacVP1 was transferred into the pQE-30 vector.

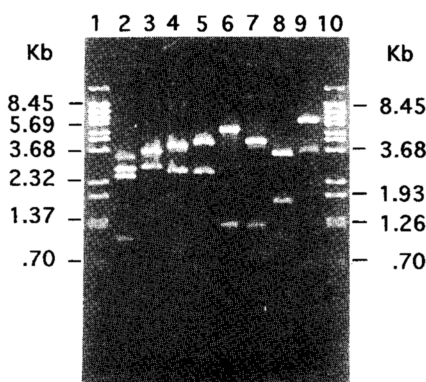


Fig. 5. Restriction enzyme analysis of pQEVP1 clone. Clone pQEVP1 was digested with restriction enzymes and electrophoresed in 1% agarose gel. Lane 1, λDNA digested with *Bst*EII. Lanes 2, pQEVP1 digested with *Xba*I; 3, with *Sac*I and *Pst*I; 4, with *Eco*RI; 5, with *Hind*III; 6, with *Nde*I and *Pst*I; 7, with *Pvu*II; 8, with *Xho*I and 9, with *Sma*I.

the cell membranes. These observations indicated that the pBacHis-VP1 subclone and the *LacZ*-HcNPV DNAs were successfully recombinated in the *S. frugiperda* cells and multiplied.

The titer of VP1-HcNPV-1 in the firstculture was about 2.0×10^5 pfu per ml at 7 days p.i., and the medium was used for the next infection inoculum.

2. Identification of VP1 protein in *E. coli* cell/pQEVP1

Whether the VP1 protein is soluble in the cytoplasm, located in cytoplasmic inclusion bodies, or secreted into the periplasmic space were determined The VP1 protein produced by pQEVP1 clone was present in soluble state and located in the cytoplasm of the cells at 23℃, 30℃ and 37℃ (Fig. 11 lanes 1, 5 and 7), however at 30℃ and 37℃ the VP1 pro-

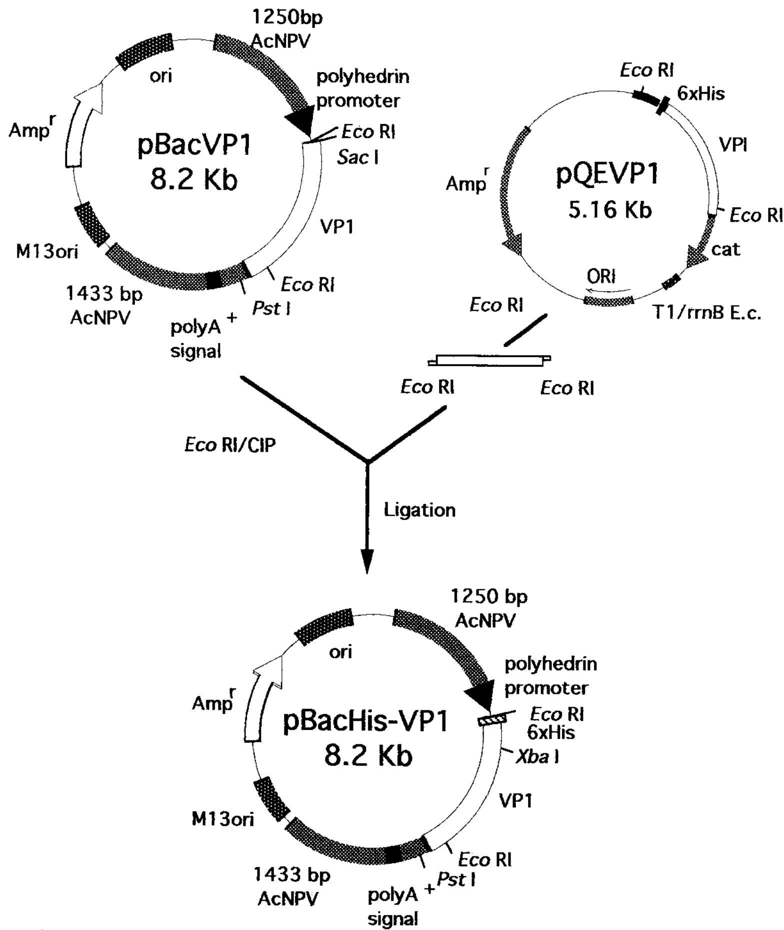


Fig. 6. Construction of pBacHis-VP1 subclone. The 6×His-tagged VP1 gene sequence in the pQEVP1 vector was transferred into the pBacVP1.

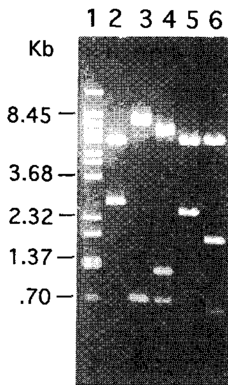


Fig. 7. Restriction enzyme analysis of pBacHis-VP1 clone. The pBacHis-VP1 DNA was digested with restriction enzymes and electrophoresed in 1% agarose gel. Lane 1, λDNA digested with *Bst*EII. Lanes 2, pBacHis-VP1 DNA digested with *Xba*I; 3, with *Bgl*II; 4, with *Pvu*II; 5, with *Eco*RI; and 6, with *Sma*I.

tein was present in insoluble state (Fig. 11 lanes 2, 4 and 8). At 30℃ and 37℃ the amounts of the insoluble VP1 protein were higher than the amounts of the soluble protein at 23℃. The arrow indicate the VP1 protein.

The VP1 protein produced by pQEVP1 in *E. coli* cells was purified by amylose affinity chromatog-raphy (Fig. 12) and identified on SDS-PAGE and Western blot (Fig. 13). The pQEVP1 clone in *E. coli* produced the VP1 protein with the molecular weight of approximately 94 kDa in the time course (Fig. 13). The 6×His-tagged VP1 protein was eluted by the chromatography with the buffers D, E and F (Fig. 12). The 6×His-tagged VP1 proteins were highly eluted in the fraction number 2 to 5 by the buffers E and D, however by the buffer F it

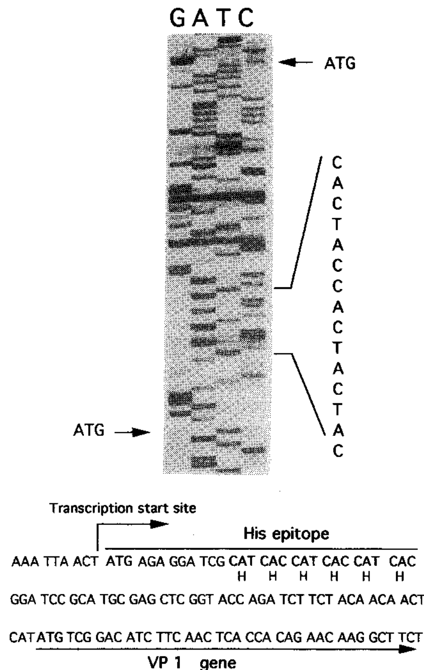


Fig. 8. DNA sequence of the cloning site of polyhedrin gene-ATG-6xHis-*Xba*I-part of VP1 gene for confirmation of right ligation. This sequence was dentermined by the dideoxy chain termination method with Hc-1 primer.

not. The 6×His-tagged VP1 protein was banded at 95 kDa, which was confirmed by Western blot using the mouse monoclonal ^{MRGS} · His antibody (Fig. 13 lane 1' to 4').

3. Identification of VP1 protein in insect cell produced by recombinant virus, VP1-HcNPV-1

S. frugiperda cells infected with the plaque-purified recombinant virus, VP1-HcNPV-1 at a m.o.i. of 5 pfu per cell for 5 days at 28℃ produced VP1 protein. Cell lysate was analyzed on SDS-polyacrylamide gels and blotted. The results revealed that a 95 kDa protein representing VP1 protein was banded (Fig. 14). The expression products by the recombinant was further analysed on Western blot using the MBP-VP1-specific antiserum. The analysis showed that 95 kDa protein was immunoreactive with the MBP-VP1 antiserum [7]. This meant that the 95 kDa protein is the VP1 protein produced by the recombinant virus VP1-HcNPV-1.

4. Activity of RNA polymerase (VP1 protein)

The crude cell lysates infected with VP1-HcNPV-1 recombinant virus strain and wild type *LacZ*-

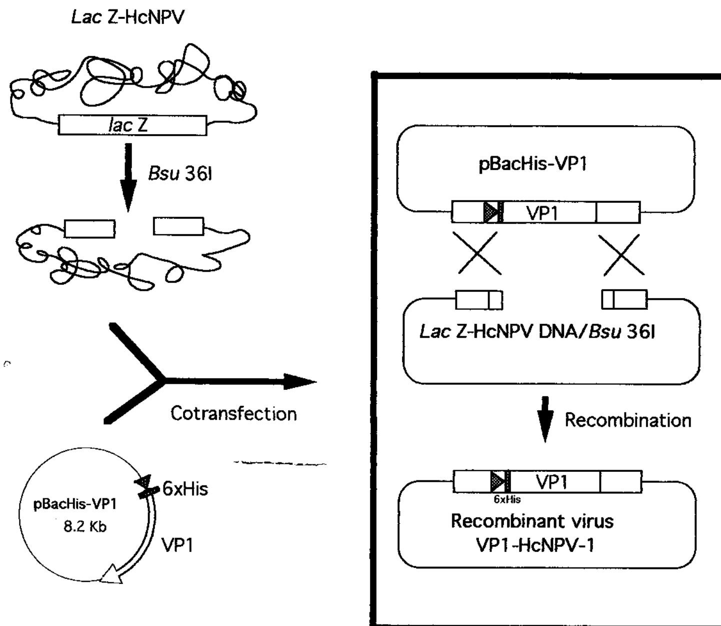


Fig. 9. Construction scheme of recombinant virus VP1-HcNPV-1 by cotransfection with *LacZ*-HcNPV and pBacHis-VP1 recombinant. *LacZ*-HcNPV was cleaved with *Bsu*36I enzyme and cotransfected with the pBacHis-VP1 clone to be recombinated with the homologous region.

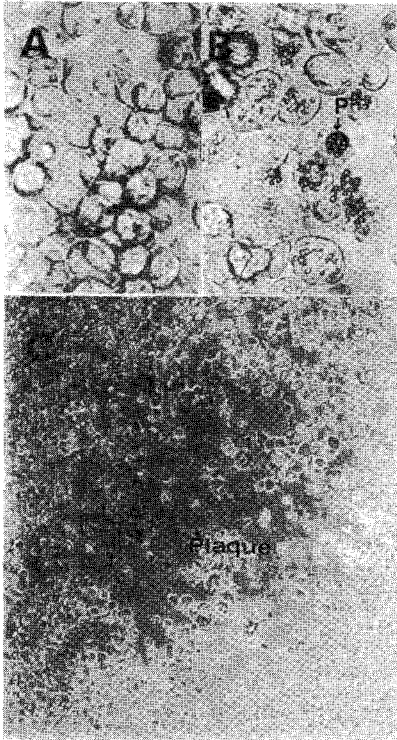


Fig. 10. **(A).** Morphology of normal *S. frugiperda* cells (×400). **(B).** Normal cells infected with HcNPV (×400). Arrow indicates polyhedral inclu-sions. **(C).** Plaque of the recombinant virus, VP1-HcNPV-1 on cell monolayers (×200). No polyhedral inclusion bodies appeared in the clear plaque and whole cells, and the shape of infected cells was hypertrophied and spheroidal shape.

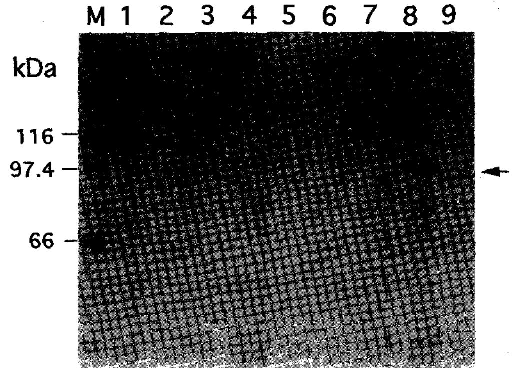


Fig. 11. Production of VP1 protein by the pQEVP1 clone. Clone pQEVP1 was cultured at 23℃, 30℃ and 37℃, and then samples were analyzed on 7.5% SDS-polyacrylamide gel. Lanes M, standard molecular weight markers; 1, supernatant-1 (soluble protein) cultured at 23℃; 2, pellet extract-1 (insoluble protein) cultured at 23℃; 3, osmotic shock extract-1 cultured at 23℃; 4, pellet extract-2 (insoluble protein) cultured at 30℃; 5, supernatant-2 (soluble protein) cultured at 30℃; 6, osmotic shock extract-2 cultured at 30℃; 7, supernatant-3 (soluble protein) cultured at 37℃; 8, pellet extract-3 (insoluble protein) cultured at 37℃; 9, osmotic shock extract-3 cultured at 37℃.

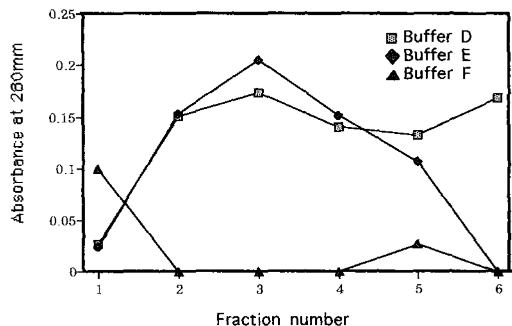


Fig. 12. Chromatogram of the 6xhis-tagged VP1 protein produced by *E. coli* M15/pQEVP1 clone. *E. coli* M15/pQEVP1 lysate was fractionated by buffers D, E, and F with Ni-NTA affinity chromatography and the eluents was measured at optical density at 280.

HcNPV (control) at 48 h were tested for RNA polymerase activity, defined as TCA-PPi precipitable radioactivity derived from α-^{32}P-CTP. Time courses of the RNA polymerase activities in the infected and uninfected lysates are shown in Fig. 15. Substantial TCA-PPi precipitable counts were detected only in the lysates of the VP1-HcNPV-1 infected cell, and these counts increased with time, leveling off after one hour of incubation. The results in Fig. 15 suggested that RNA polymerase activity was observed in the lysates of *S. frugiperda* cell infected with the recombinant virus, VP1-HcNPV-1.

DISCUSSION

The main goal of this study was to investigate the expression of the VP1 gene of the infectious pancreatic necrosis virus (IPNV) recombinated in a baculovirus, *LacZ-Hyphantria cunea* nuclear polyhedrosis virus (*Lac*Z-HcNPV).

The results described in this paper demonstrate that the baculovirus expression vector system is very useful for the high level expression of the fish virus gene and this may be first report in the use of the system. A baculovirus recombinant expressing a

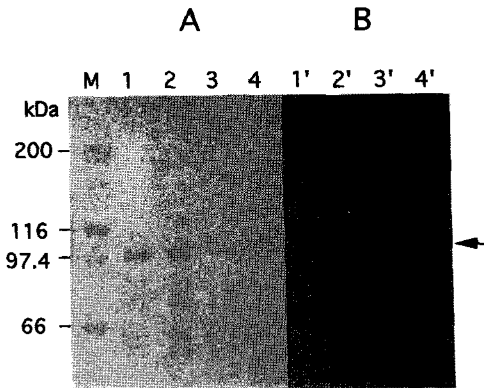


Fig. 13. SDS-PAGE and Western blot analysis of VP1 protein fraction produced by *E. coli* M15/ pQEVP1 clone. Fractions 2-5 in buffer E was concentrated and then run on the 7.5% SDS-PAGE. Arrow indicates VP1 protein. A panel: the gel stained with Coomassie blue. Lane M, standard molecular weight marker; lanes 1-4, fraction numbers 2-5 concentrated. B panel: Western blot analysis of the same gel. Lanes 1'-4', Western blot of the fraction numbers 2-5.

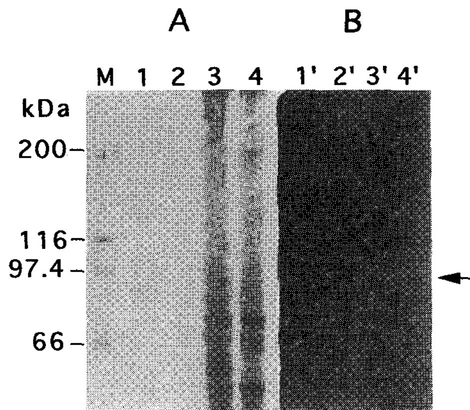


Fig. 14. SDS-PAGE and Western blot analysis for detection of VP1 protein produced by *S. frugiperda* cells infected with VP1-HcNPV-1. All sample was run on 7.5% SDS-PAGE. Arrow indicates VP1 protein. A panel. SDS-PAGE stained with Coomassie blue. Lanes M, standard molecular weight marker; 1, *E. coli* DH5α/pMal-pol lysate, 2, cell lysate infected with *LacZ*-HcNPV; 3-4, cell lysate infected with VP1-HcNPV-1. B panel. Western blot of the panel A. Lanes 5 and 6, Western blot of lanes 1 and 2; 7, Western blot of lane 3; and 8, Western blot of lane 4.

complete copy of IPNV VP1 polypeptide was constructed. The VP1 gene of RNA genome segment B cDNA of IPNV DRT strain was cloned into baculovirus transfer vectors and then constructed a re-

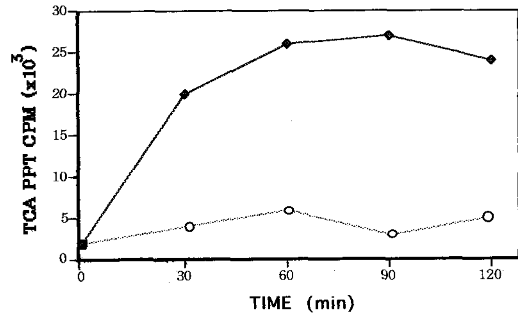


Fig. 15. Time course of RNA polymerase activity. RNA polymerase activities in the lysates from *S. frugiperda* cells infected with the VP1-HcNPV-1 recombinant (closed circle) and uninfected (open circle) were measured with liquid scintillation counter.

combinant baculovirus VP1-HcNPV-1.

The VP1 gene in the recombinant plasmid pMal-pol [7] was transfered in the transfer vector, pBacPAK9, and the resulting recombinant was named pBacVP1 clone (8.2 Kb) (Fig. 1). The VP1 gene was inserted behind the intact polyhedrin promoter of *Autographa californica* NPV in the vector plasmid. In this construction the entire polyhedrin coding region has almost been replaced by the VP1 coding sequence in such a way that the open reading frame is under the control of the polyhedrin promoter and that translation initiation will be at the ATG start codon provided by the VP1 sequence. Then the VP1 gene in the pBacVP1 was transferred in the expression vector pQE-30, and the resulting recombinant clone was named pQEVP1 (6.16 Kb) (Fig. 4). In the clone the VP1 gene is inserted behind T5 phage promoter and the 6×His region of pQE-30 vector, which makes easy purification of an expressed gene product with Ni-NTA resin colume.

To construct a recombinant baculovrius *LacZ*-HcNPV, the 6×His-VP1 gene region in the clone pQEVP1 was transferred into the *Eco*RI site of transfer vector pBacVP1, and the resulting transfer vector was named pBacHis-VP1 (Fig. 6). In this recombinant transfer vector the VP1 gene was inserted behind polyhedrin promoter and the 6×His region. Right orientation and cloning of the regions of polyhedrin promoter-ATG-6×His-*Xba*I-starting region of the VP1 gene transcription were confirmed by sequencing (Fig. 8). These results con-

firm that the VP1 gene is correctly inserted and oriented in the vector.

The host virus *LacZ*-HcNPV digested with *Bsu*361 enzyme was cotransfected and recombinated with the VP1 gene of the pBacHis-VP1 DNA in the *S. frugiperda* cells. Recombination was occurred between the homologous sequences flanking the *Bsu*361 cleaved regions of the linear *LacZ*-HcNPV and the both ends of polyhedrin gene in the vector (Fig. 9). The recombinant virus was selected and characterized by plaque assay containing X-gal. White color and no polyheda formation plaques were selected as recombinant viruses. One best selected recombinant virus was named VP1-HcNPV-1. These observations indicated that the pBacHis-VP1 subc-lone and the *LacZ*-HcNPV DNAs were successfully recombinated in the *S. frugiperda* cells and multiplied.

The VP1 protein in insect cell produced by VP1-HcNPV-1 showed 95 kDa on SDS-polyacrylamide gels and confirmed by Western blotted (Fig. 14). This meant that the 95 kDa protein is the VP1 protein produced by the recombinant virus VP1-HcNPV-1. The recombinant baculovirus was expressed high levels of the VP1 polypeptide in the insect cells. MacDonald and Dobos [22] and Nagy and Dobos [23] reported that IPNV infected cells produced VP1 protein with a molecular weight of 90 kDa. Our expressed VP1 polypeptide was migrated in SDS-PAGE as one band with apparent molecular weights of 95 kDa.

Whether the VP1 protein is soluble in the cytoplasm, located in cytoplasmic inclusion bodies, or secreted into the periplasmic space was determined. The VP1 protein produced in *E. coli* cells by pQEVP1 clone was present in soluble state and located in the cytoplasm of the cells at 23℃ (Fig. 11), however at 30℃ and 37℃ the VP1 protein was present in insoluble state (Fig. 11). At 30℃ and 37℃ the amounts of the insoluble VP1 protein were higher than the amounts of the soluble protein at 23℃. Most insoluble proteins can be solubilized in 6 M guanidine hydrochloride.

The VP1 protein produced by pQEVP1 in *E. coli* cells was purified by amylose affinity chromatography (Fig. 12) and identified on SDS-PAGE and Western blot (Fig. 13). The 6×His-tagged VP1 protein was banded at 94 kDa, which was confirmed by Western blot.

Differences in the amounts of VP1 protein were found upon expression in *E. coli* cells but overall the amounts of protein produced in the baculovirus expression system were larger than those in *E. coli*. Also the difference between the molecular weights of the VP1 protein produced in *E. coli* and *S. frugiperda* cells may could be the glycosylation of the protein in the *S. frugiperda* cells.

The crude cell lysates infected with VP1-HcNPV-1 recombinant virus strain and *LacZ*-HcNPV (control) at 48 h were examined for RNA polymerase activity, that were defined as TCA-PPi precipitable radioactivity derived from α-^{32}P-CTP (Fig. 15). Time courses of the RNA polymerase activities in the infected and uninfected lysates were measured. Substantial TCA-PPi precipitable counts were detected only in the lysates of the VP1-HcNPV-1 infected cell. The results suggested that RNA polymerase activity was observed in the lysates of *S. frugiperda* cell infected with the recombinant virus, VP1-HcNPV-1.

The expression and recombination of fish virus gene in baculovirus expression system described here may inform the basis for continued analysis of the protein activities and the gene functions.

SUMMARY

Expression of the cDNA of the VP1 gene on the genome RNA B segment of infectious pancreatic necrosis virus (IPNV) DRT strain in *E. coli* and a recombinant baculovirus were carried out.

The VP1 gene in the pMal-pol clone (Lee *et al.* 1995) was cleaved with *Xba*I and transferred into baculovirus transfer vector, pBacPAK9 and it was named pBacVP1 clone. The VP1 gene in the pBacVP1 clone was double-digested with *Sac*I and *Pst*I and then inserted just behind T5 phage promoter and the 6×His region of the pQE-30 expression vector, and it was called pQEVP1. Again, the 6×His-tagged VP1 DNA fragment in the pQEVP1 was cleaved with *Eco*RI and transferred

into the VP1 site of the pBacVP1, resulting pBacHis-VP1 recombinant. The pBacHis-VP1 DNA was co-transfected with *LacZ-Hyphantria cunea* nuclear polyhedrosis virus (*LacZ*-HcNPV) DNA digested with *Bsu*361 onto *S. frugiperda* cells to make a recombinant virus. One VP1-gene inserted recombinant virus was selected by plaque assay. The recombinant virus was named VP1-HcNPV-1.

The 6×His-tagged VP1 protein produced by the pQEVP1 was purified with Ni-NTA resin chromatography and analyzed by SDS-PAGE and Western blot analysis. The molecular weight of the VP1 protein was 94 kDa. The recombinant virus, VP1-HcNPV-1 did not form polyhedral inclusion bodies and expressed VP1 protein with 95 kDa in the infected *S. frugiperda* cells, which was detected by Western blot. The titer of the VP1-HcNPV-1 in the first infected cells was 2.0×10^5 pfu/ml at 7 days postinfection.

Acknowledgemtnt

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