Int. J. Indust. Entomol. Vol. 1, No. 1, 2000, pp. 19~23

International Journal of *Industrial Entomology*

Construction of a Novel Baculovirus *Autographa californica* Nuclear Polyhedrosis Virus Producing the Fluorescent Polyhedra

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(Received 20 December 1999; Accepted 14 July 2000)

A novel recombinant baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) producing the green fluorescent polyhedra was constructed and characterized. The recombinant virus was stably produced fluorescent polyhedra in the infected cells and the morphology of the polyhedra was nearly similar to that of wild-type AcNPV. For the production of the fluorescent polyhedra, the green fluorescent protein (GFP) gene was introduced under the control of polyhedrin gene promoter of AcNPV, by translational fusion in the front and back of intact polyhedrin gene. The recombinant baculovirus was named as CXEP. As expected, the 93 kDa fusion protein was expressed in the CXEP-infected cells. Interestingly, however, the cells infected with CXEP also showed a 33 kDa protein band as cells infected with wild-type AcNPV. The results of Southern blot analysis and plaque assay suggested that two types of baculoviruses expressing the GFP fusion protein or only native polyhedrin were formed through homologous recombination between two polyhedrin genes in the same orientation. Thus, this system can be applied for the production of recombinant polyhedra with foreign gene product of diverse interest.

Key words : Baculovirus, Polyhedra, Green Fluorescent Protein

Introduction

Baculoviruses have attractive potential as biological control agents for insect pest management owing to their host specificity, efficacy and stability (Bonning and Hammock, 1996; Wood and Granados, 1991). Baculoviruses also have been successfully used as highly efficient eucaryotic expression vectors because they possess several characteristics such as high level expression, authentic biological and immunological activity, and post-translational modification (Luckow and Summers, 1988; Maeda, 1989; O'Reilly *et al.*, 1992).

In the baculovirus, NPVs have an unique feature of producing inclusion bodies called polyhedra in the infected nucleus. Actually, the baculovirus polyhedrin generally makes up almost 20% of the total protein in the infected cells. This proportion subsequentially increases to more than 50% after the degradation of other proteins at a very late stage of infection (Granados and Federici, 1986). Polyhedrin is one of the most abundantly accumulated in virus infected-eucaryotic cells and forms the occlusion body matrix of polyhedra to 15 um in diameter (Carstens *et al.*, 1986). Polyhedra protect the numerous progeny viral particles embedded within them and are required for oral infection. Polyhedra themselves are nonessential for viral replication. Because of the importance of polyhedra in the transmission and maintenance of baculoviruses in nature, many investigators are interested in identifying genes required in polyhedra morphogenesis and studying their conformation(Chung *et al.*, 1980; Yamamoto *et al.*, 1981; Duncan *et al.*, 1983; Carstens *et al.*, 1986). In the AcNPV, amino acid essential to the normal assembly of polyhedra, a point mutation in the polyhedrin gene and polyhedra morphology mutants have been reported (Chung *et al.*, 1980; Yamamoto *et al.*, 1981). Genetic engineering technology provides a means of improving baculoviruses for use of diverse interest. Hundreds of recombinant baculoviruses using the strong polyhedrin or p10 promoter have been developed for various purposes. However, genetic engineering technology of recombinant polyhedra with foreign gene products have not yet been studied.

We have now successfully developed a novel baculov-

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irus AcNPV producing the green fluorescent polyhedra, providing a useful technology for the new applications and research possibilities. The GFP was chosen as a model protein for studying recombinant polyhedra with foreign gene product since it does not require any other substrate and can be easily assayed.

Material and Methods

Cell lines and viruses

Spodoptera frugiperda (Sf9) cells used in this study were maintained at 27°C in TC-100 medium (Sigma) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, Gibco). Wild-type Autographa californica nuclear polyhedrosis virus (AcNPV) and recombinant virus were propagated in Sf9 cells. The titer of viruses was determined by plaque assay in Sf9 cells as described previously (Summers and Smith, 1987; O'Reilly *et al.*, 1992).

Polyhedra and viral genome purification

Polyhedra and viral DNA were obtained from Sf9 cells by standard methods (O'Reilly *et al.*, 1992). Polyhedra of wild-type and recombinant AcNPV from Sf9 cells were purified by a discontinous 40 to 65% sucrose gradient centrifugation. Viral DNA was extracted from polyhedra purified from Sf9 cells. DNA was extracted with phenol after proteinase K digestion.

Recombinant transfer vector

The synthetic primers were prepared to 5'-GCCATTGTAATGAGACGCAC-3' for the 5' promoter region of AcNPV polyhedrin gene and 5'-AACTCGAGATACGCCGGACCAGTGAAC-3' for the 3' coding region of AcNPV polyhedrin gene which added *Xho*I site for the cloning and removed termination codon TAA. Two primers were used to amplify a 903 bp polyhedrin gene region from AcNPV (All PCR reactions comprised 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min). The amplified product was digested with *Eco*RV and *Xho*I, and inserted into the *Eco*RV and *Xho*I sites of pBacPAK8 to yield pBacPAK8-AcPH. The polyhedrin gene was amplified from AcNPV by two synthetic primers, 5'-AACTCGAGATGCCGGATTATTCATACC-3' for the translational start sequence region containing the *Xho*I site and 5'-CCGGCGTATTAAGAATTCAA-3' for the 3' coding region containing the *Eco*RI site. The amplified polyhedrin gene was digested with *Xho*I and *Eco*RI, and introduced into the *Xho*I and *Eco*RI sites of pBacPAK8-AcPH to yield pAcDPH. Finally, the synthetic primers were prepared to 5'-AACTCGAGATGAGTAAAGGAGAA-3' for the translational start region of the GFP gene containing the *Xho*I site and 5'-AACTCGAGTTTGTATAGTTCATCC-3' for the 3' coding region of the GFP gene which also added *Xho*I site and deleted termination codon TAG. Two primers were used to amplify the GFP gene from pGFP (Clontech). The amplified the GFP gene was digested with *Xho*I, and inserted into the *Xho*I site of pAcDPH to yield pCXEP.

Construction of Recombinant AcNPV.

The cell culture dish (35-mm diameter) seeded with 1.0×10^6 Sf9 cells was incubated at 27°C for 1 hr to allow the cells to attach. One microgram of BacPAK6 viral DNA (Clontech, USA), 5 μg of pCXEP plasmid DNA in 20 mM HEPES buffer and sterile water to make a total volume of 50 μl were mixed in a polystyrene tube. Fifty μl of 100 μg/ml Lipofectin™ (Gibco, USA) were gently mixed the DNA solution, and the mixture was incubated at room temperature for 30 min. The cells were washed twice with 2 ml serum-free TC-100 medium (Gibco, USA). Serum-free TC-100 (1.5 ml) was added to each dish. The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubating at 27°C for 5 hr, 1.5 ml TC-100 containing antibiotics and 10% FBS was added to each dish and the incubation at 27°C continued. At 5 days postinfection (p.i.), the supernatant was harvested, clarified by centrifugation at 2,000 rpm for 5 min, and stored at 4°C before plaquing on Sf9 cells. To plaque purify recombinant AcNPV, 1.5×10^6 Sf9 cells were seeded per well on a 6-well plate and the supernatant was added to each well. Recombinant AcNPV was plaque purified in Sf9 cells (O'Reilly *et al.*, 1992).

Microscopy

Microscopy of Sf9 cells infected with recombinant virus was performed using a light and fluorescent microscope (Axiophot Universal Microscope, Zeiss).

SDS-polyacrylamide gel electrophoresis (PAGE)

Sf9 cells were mock-infected or infected with the wild-type AcNPV and recombinant baculovirus in a 35-mm diameter dish (1×10^6 cells) at a MOI of 5 PFU per cell. After incubation at 27°C, cells were harvested at 2, 3, and 4 days p.i. For SDS-PAGE of cell lysates, uninfected Sf9 cells and cells infected with virus were washed twice with PBS (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and mixed with a protein sample buffer. The total cellular lysates were performed on a 10% polyacrylamide separating gel with a 3% stacking gel containing SDS as described by Laemmli (1970).

Southern blot analysis

The viral DNAs were completely digested with restric-

tion enzymes and electrophoresed on a 0.8% agarose gel. The DNAs were transferred to nylon membrane by capillary transfer. The GFP and polyhedrin genes were used as a probe. Probe DNA lebelling and detection of immobilized target DNA were carried out according to method recommended by supplier using Southern-light™ chemiluminescent labeling and detection system (TROPIX, Inc.).

Plaque assay

Sf9 cells were seeded into 6 well tissue culture plates at a density of 1×10^6 cells/well and allowed to attach for 1 hr. Cells were inoculated at a MOI of 5 PFU per cell with the recombinant virus, CXEP. After 2 hr, the inoculum was removed and the monolayer was overlaid with 2 m*l* of 1.5% SeaPlaque agarose (Mandel) in medium containing 50 μg/m*l* of gentamycin. Plaques were observed to six days p.i..

Results

Construction of recombinant AcNPV

The baculovirus transfer vector summarizing map used to generate recombinant virus producing the green fluorescent polyhedra was described in Fig. 1A. The GFP gene was translationally fused in the front and back of polyhedrin gene of pAcDPH to yield pCXEP. The fusion gene was introduced under the control of AcNPV polyhedrin promoter. Genomic DNA of wild-type AcNPV was cotransfected with baculovirus transfer vector, pCXEP into Sf9 cells. The supernatant was collected at 5 days p.i., reinfected into a monolayer of Sf9 cells, and recombinant virus was plaque purified. The recombinant virus was named as CXEP.

To determine the formation of recombinant polyhedra, cells infected with CXEP were observed by light and fluorescent microscope (Fig. 1B). Interestingly, cells infected with CXEP produced the green fluorescent polyhedra and the fluorescence of The GFP was only detected within the nuclei of cells. Furthermore, the morphology of the polyhedra was nearly similar to that of wild-type AcNPV.

Expression of the fusion gene in insect cells

In order to examine the expression of fusion gene by recombinant virus in Sf9 cells, the protein synthesis in Sf9 cells infected with CXEP was analyzed by SDS-PAGE (Fig. 2). Wild-type AcNPV-infected cells showed a pattern of protein synthesis typical of AcNPV infected cells. Fusion protein band was not detected in cells infected with wild-type AcNPV or from mock-infected cells. The fusion protein expressed by the fusion gene was present as a band of about 93 kDa in cells infected with recombinant virus, CXEP, which was absent in cells infected with wild-type AcNPV. However, the cells infected with CXEP also showed a 33 kDa protein band as cells infected with wild-type AcNPV.

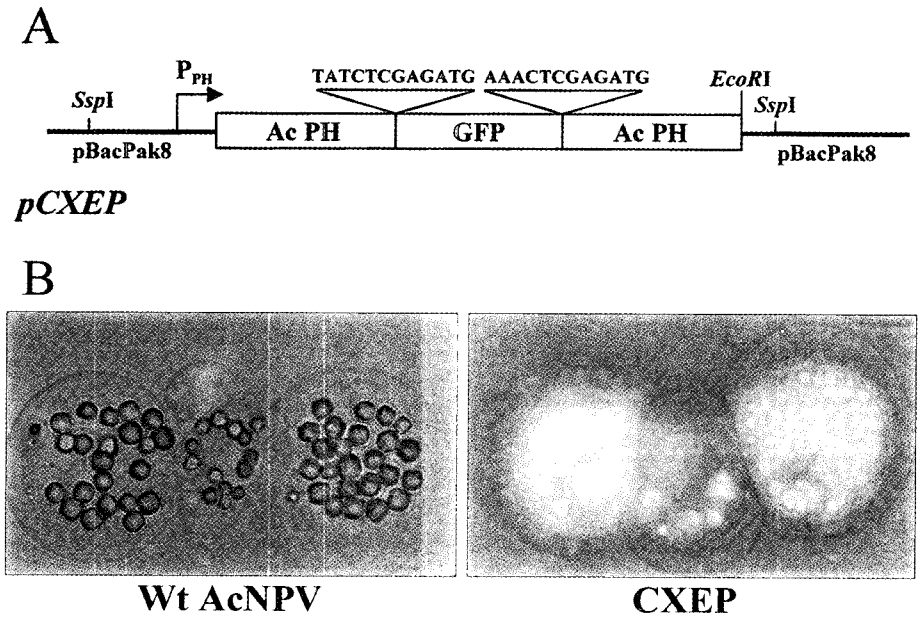

Fig. 1. The baculovirus transfer vector map used to generate recombinant virus (A) and microscopy of Sf9 cells infected with recombinant virus (B). the GFP gene was introduced under the control of polyhedrin gene promoter of AcNPV, by translational fusion in the front and back of intact polyhedrin gene to yield pCXEP. Sf9 cells infected with wild-type AcNPV and CXEP were observed by light and fluorescent microscope (×1,000).

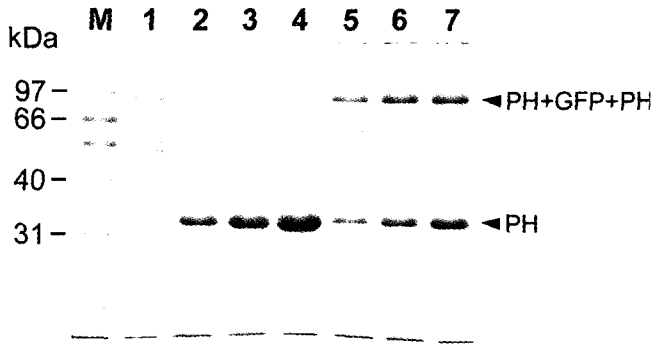

Fig. 2. SDS-PAGE analysis of the fusion protein expression of recombinant virus CXEP in Sf9 cells. Sf9 cells were mock infected (lane 1) or infected with wild-type AcNPV (lanes 2, 3 and 4) and CXEP (lanes 5, 6 and 7) at a MOI of 5 PFU per cell. Cells were collected at 2 (lanes 2 and 5), 3 (lanes 3 and 6) and 4 (lanes 4 and 7) days p.i. Total cellular lysates were subjected to 10% SDS-PAGE. Polyhedrin and fusion protein bands are indicated on the right of panel. Molecular weight standards were used as size marker (lane M).

Homologous recombination of CXEP in insect cells

To test the possibility of the homologous recombination (Watson *et al.*, 1987) between two polyhedrin genes in the same orientation, genomic DNA from recombinant baculovirus CXEP was extracted and analyzed by Southern hybridization (Fig. 3). Hybridization of *Sph*I or *Ssp*I digested CXEP DNA with probe comprising the GFP gene showed a singl band of about 6.5 or 3.0 kb, confirming that CXEP genome contains intact the GFP gene (Fig. 3B). As expected, the GFP gene band was not detected in wild-type AcNPV. In contrast, use of the polyhedrin gene as probe revealed different band pattern of CXEP (Fig. 3C). Especially, Southern hybridization of an *Ssp*I digest of CXEP revealed a 1.6 kb band containing polyhedrin gene as that of wild-type AcNPV. These results showed that recombinant baculovirus was mixed with two types, indicating that recombinant baculoviruses expressing fusion protein or only polyhedrin were constructed by homologous recombination in insect cells. To support this interpretation, the 1.6 kb *Ssp*I fragment of CXEP and wild-type AcNPV detected by the polyhedrin gene probe was cloned and their structure confirmed by sequence analysis (data not shown). The result showed that the 1.6 kb *Ssp*I fragment of CXEP was different from that of wild-type AcNPV, which does not possess *Eco*RI site. Accordingly, the result of plaque assay about recombinant virus CXEP also revealed that two proteins, fusion protein and only polyhedrin, were respectively produced from two recombinant baculoviruses.

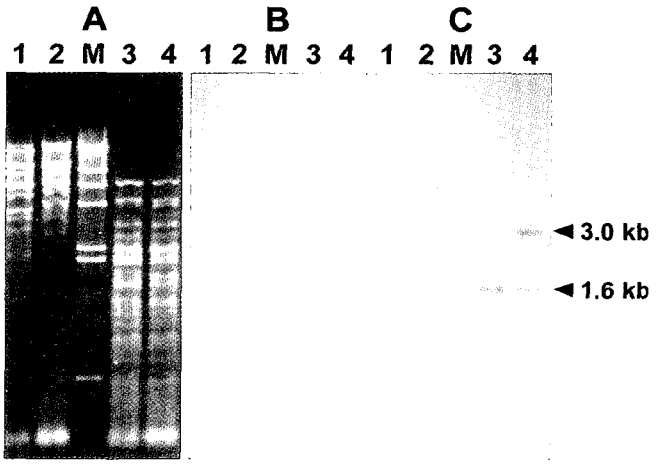

Fig. 3. Restriction analysis (A) and Southern hybridization (B and C) of baculovirus genomes. Wild-type (lanes 1 and 3) and CXEP (lanes 2 and 4) DNAs were completely digested with *Sph*I (lanes 1 and 2) and *Ssp*I (lanes 3 and 4), and electrophoresed on a 0.8% agarose gel. The DNAs were transferred to nylon membrane by capillary transfer. The probes were GFP (B) and polyhedrin genes (C) as described in the Materials and Methods. Size standards were lambda DNA cleaved with *Hin*dIII (lane M).

Discussion

We have tried construction of a novel recombinant AcNPV producing polyhedra with foreign gene product. In the previous report (Je *et al.*, 1999), we constructed recombinant baculoviruses producing fusion protein with polyhedrin and GFP although these viruses were failed to

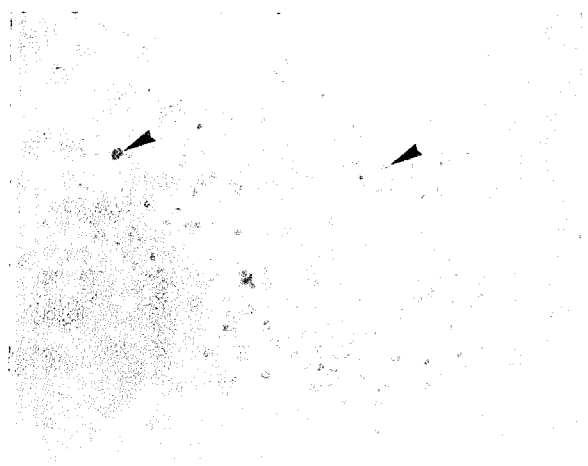

Fig. 4. Microscopy of Sf9 cell plaque infected with recombinant virus CXEP. Sf9 cells were inoculated at a MOI of 5 PFU per cell with CXEP. After 2 hr, the inoculum was removed and the monolayer was overlaid with 1.5% SeaPlaque agarose as described in the materials and methods. Plaques were observed by light and fluorescent microscope (×200) at six days p.i. Arrows indicate recombinant AcNPV expressing native polyhedrin.

form mature polyhedra in the infected cells. In the AcPOLGFP-infected cells, however, polyhedra-like granular particles were apparently observed within the nuclei. This result suggests that the polyhedrin in the fusion protein has any effect for the presence of the polyhedra-like particles with fluorescence. In the second approach, the AcGFP or the AcPOLGFP and the wild-type AcNPV were coinfected into Sf9 cells. In this case, the striking difference between the coinfected cells with AcPOLGFP or AcGFP and wild-type AcNPV was that cells coinfected with the AcPOLGFP and the wild-type AcNPV were seen fluorescent polyhedra in the nuclei, whereas cells coinfected with the AcGFP and the wild-type AcNPV were not seen (data not shown). Actually, the fact that coinfection with the AcPOLGFP and wild-type AcNPV produces fluorescent polyhedra suggests that the result could provide clues about the formation of recombinant polyhedra with foreign gene product.

From these data we learned that the native polyhedrin and the polyhedrin in the front of the fusion protein have an important role in the formation of recombinant polyhedra. For the stable production of the recombinant polyhedra, both native polyhedrin and fusion protein with GFP and polyhedrin need to be expressed in the same cell. In this report, we achieved this double expression with a third approach. The strategy was to create a recombinant virus CXEP described in Fig. 1A. The CXEP was greatly improved in the homologous recombination between two polyhedrin genes in the same orientations. We analyzed genomic DNA from the CXEP with Southern hybridization using polyhedrin gene probe to identify homologous recombination (Fig. 3C). Thus, cells infected with the CXEP produced stably fluorescent polyhedra which were similar to morphology and size of the wild-type AcNPV polyhedra.

On the basis of our current knowledge of this CXEP system, the most likely mechanism of the assembly of recombinant polyhedra is that the fluorescent polyhedra are formed by coassembly both the fusion protein with GFP and the native polyhedrin coexpressed in the infected cell, a possibility which is due to the interaction between polyhedrin present in the fusion protein and native polyhedrin during polyhedra occlusion processing.

In conclusion, our approach has shown that the production of recombinant polyhedra with foreign gene product is possible. Thus, the recombinant polyhedra technology in the baculovirus should be expected to open a variety of new applications and research possibilities.

Acknowledgments

This work was supported by the Brain Korea 21 project

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