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Abstract. An insect cell line NISES-AnPe-428 (AnPe) derived from embryos of tussah (*Antheraea pernyi*) was transfected with DNA genome of *A. pernyi* nucleopolyhedrovirus (AnpeNPV), and infectious AnpeNPV particles were recovered and accumulated in the culture supernatant. The AnpeNPV derived from transfected viral DNA and its three plaque-purified clones showed the similar pathogenicity to both *A. pernyi* pupae and AnPe cells, although the restriction endonuclease analysis of viral genome revealed that there were several differences in electrophoretic pattern of *Hind*III fragments among the clones. In the cross infection experiment, BmN4 cells, derived from mulberry silkmoth (*Bombyx mori*) and susceptible to *B. mori* NPV (BmNPV), did not support AnpeNPV multiplication, while AnPe cells did not support BmNPV multiplication, indicating that difference in host range specificity between the two NPVs would be determined at cellular level. Altogether, we have succeeded to establish a novel system for the infection and multiplication of AnpeNPV, which forms the basis for constructing the AnpeNPV expression vector system to produce large amounts of recombinant proteins in *A. pernyi* pupae.
Keywords : AnpeNPV, tussah cell line, transfect, plaque, host range

Introduction

Recent years, the research on obtaining exogenous gene products by baculovirus expression vector system (BEVS) has been attached more importance in both basic and applied biology. In the comparison between *E. coli* expression system and BEVS, exogenous gene products in BEVS are not only produced in large amounts safely, but also processed with eukaryote-specific posttranslational modifications, such as glycosylation, phosphorylation and proteolytic cleavage (MAEDA, 1993). Because of these modification mechanisms, eukaryotic proteins produced in BEVS are structurally and functionally similar to the natural counterparts, that is an advantage of BEVS. In 1983, SMITH et al. first established *Autographa californica* nucleopolyhedrovirus (AcNPV) vector system, and in 1985, MAEDA et al. established *Bombyx mori* NPV(BmNPV) vector system. Now these two BEVS are used extensively in the world.

Tussah (*Antheraea pernyi*) is a giant wild silkmoth, which overwinters by pupal diapause and has been considered as an ideal host insect for BEVS (ZHANG, et al, 1990; 1992a, b). In order to construct a novel BEVS

using AnpeNPV/tussah pupae, we have established the infection and multiplication system of AnpeNPV using cultured tussah cells in this study. Clones of AnpeNPV were purified by plaque assay and their properties were analyzed. The results are as follows.

Materials and Methods

Cell, Virus and Enzyme

A tussah cell line used in the experiment was NISES-AnPe-428 (AnPe) derived from embryos of *A. pernyi* (INOUE and HAYASAKA, 1995). A *B. mori* cell line, BmN4 (MAEDA, 1989) was also used. The two cell lines were cultured continuously at 27 ℃ in MGM-448 medium (MITSUHASHI, 1984) supplemented with 10% fetal bovine serum (FBS).

AnpeNPV used was isolated from infected tussah pupae collected in Feng Huang Cheng area, Liao Ning province, China. BmNPV T3 was a gift from Dr. S. Maeda.

All restriction endonucleases used were purchased from NIPPON GENE.

Extraction of AnpeNPV DNA

Tussah pupae infected with AnpeNPV were homogenized and filtered through four layers of gauze. The filtrate was centrifuged at 3000 rpm for 15 min, and then the pellet was suspended in the distilled water and filtered again through four layers of gauze. The filtrate and percoll (Pharmacia) (pH7.3) were mixed at 3 : 5, and centrifuged at 25000rpm using Beckman SW27 rotor for 30 min at 15℃. The precipitate containing AnpeNPV polyhedra was resuspended in the distilled water, then centrifuged at 3000rpm for 15 min and the purified polyhedra were obtained as the pellet. After dissolving the purified polyhedra in the alkaline solution (0.1 M Na_2CO_3, 0.05 M NaCl) for about 30 min at room temperature, the solution was centrifuged at 3000rpm for 5 min at 4℃. The supernatant was applied on a 20% to 50% sucrose gradient and centrifuged at 15000rpm using Beckman SW27 rotor for 30 min at 15 ℃. The white band containing AnpeNPV virions was collected by injector, diluted with TE buffer (pH8.0) and centrifuged at 25000rpm using Beckman SW 41Ti rotor for 60 min at 15 ℃. The precipitate of purified virions was suspended in the lysis buffer (TE buffer, pH 8.0, containing 1mg/ml of proteinase K and 1% SDS) and incubated for 2 h in the waterbath at 65 ℃. After the incubation, viral DNA was extracted from the lysis buffer by treating with phenol, phenol/chloroform (1 : 1) and chloroform, twice respectively. Finally, AnpeNPV DNA was obtained by dialyzing extracted aqueous DNA solution against TE buffer (pH 8.0) for 16 h at 4℃. The viral DNA solution was stored at 4℃ until use.

Transfection of AnpeNPV DNA into cultured tussah cells

Transfection mixture was made by adding 0.3 μg AnpeNPV DNA and 8 μl Lipofectin reagent (Gibco BRL) to 1ml serum-free TC-100 medium (MAEDA, 1989) and applied to AnPe cells (3×10^5 cells) seeded in the 6-well plate. After incubation for 7 h at 27 ℃, the mixture was replaced with 2 ml MGM-448 medium with 10% FBS and the transfected cells were cultured for 7 days at 27 ℃. Then the culture supernatant containing AnpeNPV virions were collected and used for infection to cultured cells and *A. pernyi* pupae as well as for cloning AnpeNPV.

Cloning AnpeNPV by plaque assay

In each well of 6-well plate, AnPe cells (2×10^5 cells) were seeded and attached onto the basement. Then the culture medium was replaced with 0.4 ml of virus inoculum, which was made by diluting the culture supernatant containing AnpeNPV virions using Carlson's solution (CARLSON, 1946). After 60 min of incubation at room temperature, the virus inoculum in each well was discarded and 2.5 ml MGM-448 medium containing 10% FBS and 0.75% Sea Plaque Agarose (FMC) was added. The cells were cultured for one week at 27℃ and occlusion-positive plaques formed by AnpeNPV infection became visible. Three different plaques were separately aspirated by Pasteur pipette and dispersed in Carlson's solution, which were further added to AnPe cell cultures to amplify the plaque-purified

clones and one week later the culture supernatants were stored at –80℃ as the virus stocks.

Results

Infection and multiplication of AnpeNPV in cultured tussah cells

First, occluded virions (OVs) of AnpeNPV purified from polyhedra were infected to AnPe cells. One week post-infection, however, neither cytopathic effects (CPEs) nor formation of polyhedra were observed by microscopic observation, indicating AnPe cells are not susceptible to OV infection.

Next, the AnpeNPV DNA genome was transfected to AnPe cells, and one week later a number of nuclear polyhedra were observed within some cells. Two hundreds microliters of culture supernatant one week post-transfection were further added to AnPe cell culture. Then the number of infected cells with polyhedra in the nuclei gradually increased and almost all cultured cells were infected one week post-infection, clearly demonstrating that AnPe cells support infection and multiplication of budded virions (BVs) of AnpeNPV. Thus, the system for AnpeNPV infection and multiplication using cultured tussah cells had been established.

Cloning AnpeNPV

In order to establish the genetically homogeneous wild type AnpeNPV clone, plaque assay on AnPe cells was performed using a series of 10-fold dilutions of the infected culture supernatant containing AnpeNPV BVs. From 10^{-5} dilution, three AnpeNPV clones are isolated from different occlusion-positive plaques. These clones were subjected to additional two cycles of plaque assays and named clones A, B and C, respectively.

Infection of cloned AnpeNPV to diapausing tussah pupae

The virus stocks (ca. 1×10^{7} pfu/ml) of three AnpeNPV clones (A, B and C) and non-cloned original virus (O) were diluted 10-fold with Carlson's solution and injected (100 μl/pupa) to abdomen of diapausing tussah pupae. For each virus infection, 8 tussah pupae (4 females and 4 males) stored at 4℃ were used. As the mock infection, the same volume of Carlson's solution was injected. Then the pupae were incubated at 25℃ and observed every 5 days.

From 10 days post-injection, dead pupae with liquefied tissues and dark-colored vertex plate appeared and until 25 days post-infection all pupae injected with each virus died out (Fig. 1). It was confirmed that these dead pupae were all infected with AnpeNPV by observing large numbers of polyhedra in the liquefied tissues under microscope. In contrast, 8 pupae of mock infection did not show any disease symptoms until 25 days post-infection.

Restriction endonuclease analysis of AnpeNPV DNA genomes

In order to compare the DNA genomes of three AnpeNPV clones and non-cloned original virus, DNA genomes of each virus was cleaved with *Hind* Ⅲ and electrophoresed on an agarose gel (Fig.2). There are several differences in electrophoretic profile of *Hind* Ⅲ fragments among AnpeNPV clones A, B and C and original virus, suggesting that original virus was a mixture of genetically heterogeneous viruses and the three clones were segregated from the mixture by plaque assay.

Host range analysis of AnpeNPV

Cross infection experiment was performed using two NPVs, AnpeNPV clone A and BmNPV T3, and two cell lines, AnPe and BmN4 cells. It was found that BmN4 cells, which was highly susceptible to BmNPV, did not support AnpeNPV multiplication, while AnPe cells did not support BmNPV multiplication, indicating that difference in host range specificity between the two NPVs would be determined by unknown mechanisms at cellular level.

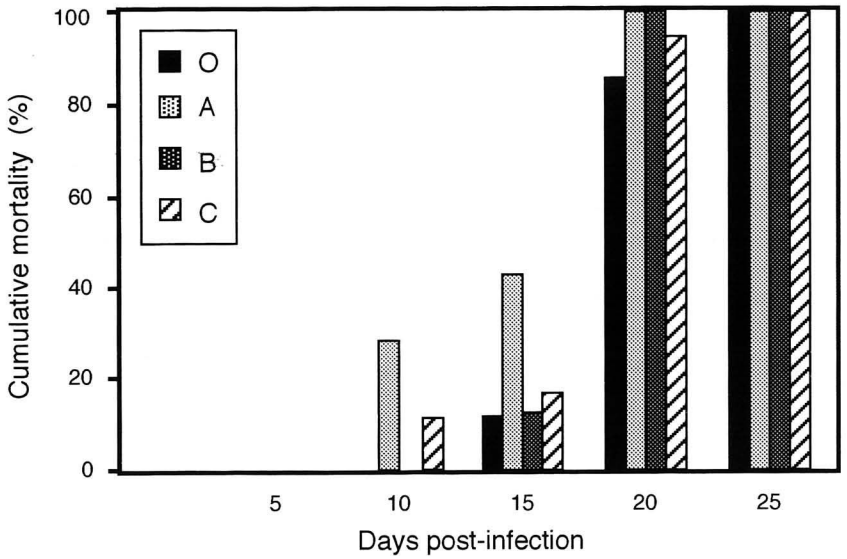

Fig. 1 Death rate of diapausing tussah pupae injected with AnpeNPV. O, non-cloned original virus; A, clone A; B, clone B; C, clone C.

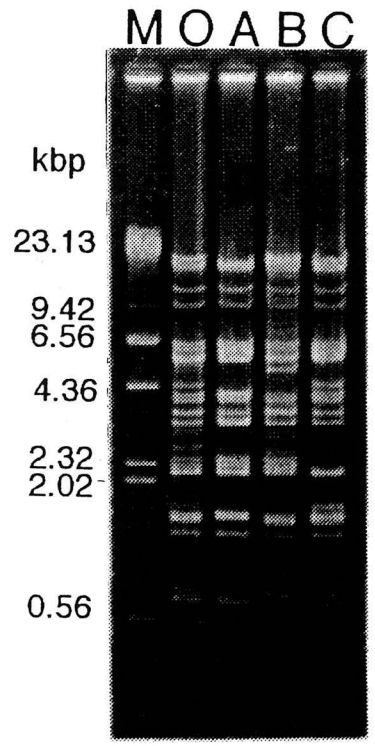

Fig. 2 Restriction endonuclease analysis of plaque-purified AnpeNPV clones. Purified viral DNAs of clones A, B and C as well as non-cloned original virus (O) were cleaved with *Hin*dIII and electrophoresed on a 0.8% agarose gel. Lane M shows λ /*Hin*dIII DNA size marker and length in kbp of each fragment is indicated at the left side.

Discussion

The research formerly showed that BmNPV infects *B. mori* larvae, while AnpeNPV infects tussah larvae, and that the two NPVs never cross-infect respective host insects (LIU, 1982). The similar host specificity in virus infection was also observed in host insect cell lines, BmN4 and AnPe cells, indicating that the cellular mechanisms which determine the host range of each virus might be common in both host insects and cultured cells.

LIU and HE (1988) reported that *B. mori* larvae were infected with BmNPV by subcutaneous injection of the viral DNA genome and, for tussah, both primary cultured ovarian cells and pupae were also infected with AnpeNPV by inoculation and subcutaneous injection of the viral DNA genome, respectively. In this study, infectious AnpeNPV was successfully recovered from AnPe cells transfected with the viral DNA genome using Lipofectin reagent. Thus a novel AnpeNPV infection and multiplication system based on the cultured tussah cells was established. In addition, when AnPe cells were infected with AnpeNPV OVs derived from polyhedra instead of BVs, virus multiplication did not occur, although the mechanism of this phenomenon is not yet known.

As the first step for constructing exogenous gene expression system in which tussah pupa and AnpeNPV are used as host and vector, AnpeNPV were purified by plaque assay, because the use of genetically heterogeneous virus stock might result in unstable and unreproducible gene expression. Among three AnpeNPV clones (A, B and C) and non-cloned original virus, electrophoretic profiles of *Hin*d Ⅲ fragments of DNA genome were compared and found to be slightly different one another (Fig.2). Precise analysis indicated that the *Hin*dⅢ fragments of original virus contain all of the fragments specific to each clone. The results revealed that original virus was a mixture of AnpeNPVs with heterogeneous DNA genomes, which may reflect a variety of mutations occurred and accumulated in the field population of AnpeNPV for a long time. Further analyses including genome DNA sequencing are needed to characterize each mutation.

The experiment on AnpeNPV infection to diapausing tussah pupae showed that all of the three clones multiplicate and kill the infected pupae at the same speed as non-cloned original virus (Fig. 1), proving that pathogenicity of each clone was not reduced, in spite of the differences in viral DNA genome. Pathogenicity is a very important viral property to establish the BEVS, because virus with strong pathogenicity is expected to multiplicate and express exogenous genes quickly and enormously. In this research, the three AnpeNPV clones with strong pathogenicity to tussah pupae were obtained. Thus, the combination of AnpeNPV clones, AnPe cells and diapausing tussah pupae forms the basis for constructing recombinant AnpeNPVs and establishing a novel BEVS using AnpeNPV and tussah pupae.

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