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Continuous Cell Line from Pupal Ovary of *Perina nuda* (Lepidoptera: Lymantriidae) That Is Permissive to Nuclear Polyhedrosis Virus from *P. nuda*

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A continuous cell line, designated NTU-PN-HH, was established from the pupal ovary of *Perina nuda* Fabricius (Lepidoptera: Lymantriidae). The cells have been through more than 300 passages during 4 years in TNM-FH medium supplemented with 10% FBS, at a constant temperature of 28°C. The cell line consists of four major morphologic types: polymorphic cells, spindle-shaped cells, round cells, and squamous cells. The characterization of this cell line showed that NTU-PN-HH is a newly established cell line. It is the first cell line that is permissive to PnNPV (*P. nuda* multiple nuclear polyhedrosis virus).

KEY WORDS: ***Perina nuda;*** **NTU-PN-HH cell line; PnNPV.**

INTRODUCTION

Many Lepidopteran cell lines have been established since 1962 (Grace, 1962; Hink, 1970, 1980), several of which have been successfully used to multiply nuclear polyhedrosis virus (NPV) (Wu *et al.,* 1989). These virus-permissive cell lines have contributed significantly to pest control (Smiths and Vlak, 1988) and to the production of recombinant proteins (Fraser, 1989; Maeda *et al.,* 1985; Smith *et al.,* 1983; Wu *et al.,* 1989). Over 600 isolates of baculoviruses have been found, but the study of most of these have been limited by a lack of susceptible cell lines on which to propagate these viruses (Gelernter and Federici, 1986). To facilitate further study of baculoviruses, it is necessary to establish an insect cell line of high viral susceptibility.

Perina nuda (Fabricius) is a major pest of banyan (*Ficus* spp.), an important garden tree in Taiwan and mainland China. The larvae of *P. nuda* are external feeders on foliage and cause the destruction of tree leaves. The epizootic disease occurs every year from spring to early summer in Taiwan and mainland China. The key pathogen was found to be baculovirus PnNPV (*P. nuda* multiple nuclear polyhedrosis virus) (Su *et al.,* 1983; Lo *et al.,* 1990; Wang and Tsai, 1995). Because there were no permissive cell lines for PnNPV, the study of this virus has been limited to morphological descriptions. We have established a permissive cell line derived from pupal ovary of *P. nuda,* which represents a great hope for extensive study of PnNPV at the pathologic, cellular, and molecular levels.

MATERIALS AND METHODS

Primary Culture and Subculture

Larvae of *P. nuda* were collected from the campus of National Taiwan University, and fed banyan leaves at 25°C. The larvae were allowed to go through the pupal stage, after which the pupae were collected and sterilized with a 10% Clorox solution and 70% iodine alcohol. The ovaries were removed with a fine forceps and a pipet, and incubated at 28°C in TNM-FH medium (Hink and Strauss, 1976) containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1.25 μg/ml fungizone. The medium was supplemented with 10% fetal bovine serum (FBS) that had been inactivated at 56°C for 30 min.

The cells were subcultured when approaching confluency. During the first 25 passages, the cells adhering to the bottom of the flask had to be removed with a rubber policeman, but thereafter could be resuspended by vigorous agitation. When subculturing, 1 ml of suspended cells were transferred to a new 25 cm^2 flask containing 4 ml of fresh media plus supplements. From the initial subculture to the 40th passage, the interval between subcultures ranged from 1 to 3 weeks, depending on the growth rate of the cells. After the 49th passage, the cells propagated rapidly, and thereafter the interval between passages was 4 days. The resulting cell line has been designated NTU-PN-HH.

Susceptibility of Virus

The following viruses were used to test the viral susceptibility of NTU-PN-HH cells: AcNPV (*Autographa*

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californica NPV) and HzNPV (*Heliothis zea* NPV), kindly supplied by Dr. M. J. Fraser of Notre Dame University; BmNPV (*Bombyx mori* NPV) collected from infected Bm-N cells or infected larvae of *B. mori* and PnNPV collected from infected larvae of *P. nuda.*

These four viruses were used to examine the viral susceptibility of three other cell lines as a positive control for virus infectivity (IPLB-SF-21AE, Bm-N, and IPLB-HZ-1075 [*H. zea* cell line]) (Goodwin *et al.,* 1982; Maeda, 1989; Vaughn *et al.,* 1977). AcNPV, HzNPV, and BmNPV were obtained from their permissive cell lines, IPLB-SF-21AE, IPLB-HZ-1075, and Bm-N, respectively. The semiconfluent tested cells (log-phase cells) of NTU-PN-HH were inoculated with the filtered homogenate of infected *P. nuda* larvae or the filtered culture medium of the NPV-infected cells of IPLB-SF-21AE, IPLB-HZ-1075, and Bm-N. After 1 hr of adsorption, the viral solution was discarded and the cells were incubated in fresh TNM-FH medium at 28°C. The cytopathic effect (CPE) was observed and documented. The virus titer was determined by the end-point dilution method ($TCID_{50}$ analysis) (Summers and Smith, 1988).

Electron Microscopy

The infected cells were scraped from the surface of the plastic flasks and sedimented at 900 rpm. The medium was discarded and the pellets were fixed in 2.5% glutaraldehyde in pH 7.2 phosphate buffer at 4°C for 3 hr and postfixed in 1% OsO_4 in the same buffer for 2 hr. The pellets were then dehydrated in an alcohol gradient series and embedded in Spurr Epon. Thin sections were cut on a Reichert OMU 3 ultramicrotome and stained with uranyl acetate and lead citrate. The photomicrographs were made with a Hitachi H7100 electron microscope at 100 kV.

Chromosome Number

The NTU-PN-HH chromosome spreads were prepared by treating 5 ml of log-phase cultures (2×10^6 cells/ml) with 0.06 μg/ml demecolcine (Sigma, D-6279) for 2 hr at 28°C. The cells were dispersed and centrifuged (900 rpm) for 5 min, resuspended for 10 min in a hypotonic solution of normal saline and distilled water in a 1:4 dilution, and then fixed in 3:1 methanol:glacial acetic acid for 20 min. The fixed cells were dropped vertically onto the slides. After air drying, the cells were stained with Giemsa stain for 30 min and the chromosome number was counted under a microscope.

Growth Rate of NTU-PN-HH Cells

The NTU-PN-HH cells were seeded in 25 cm^2 flasks, about 1×10^6 cells each, and cultured with 0, 5, 10, and 20% FBS supplementation at 28°C, and at 4, 20, 28, and 37°C with 10% FBS supplementation. The cultured cells were counted with a hemocytometer every 24 hr for 6 days.

Isozyme Analysis

The confluent cells of NTU-PN-HH, IPLB-SF-21AE, and IPLB-LD-652Y (*Lymantria dispar* cell line) (Goodwin *et al.,* 1978) were collected and centrifuged at 900 rpm for 10 min at 4°C. The pellets were washed twice in phosphate-buffered saline (PBS) and then resuspended in a grinding buffer (0.125 *M* Tris, 0.046 *M* citric acid, 10% sucrose, 1% Triton X-100, and 0.02 m*M* bromophenol blue). The cells were frozen in liquid nitrogen and thawed (37°C) three times and then cell lysates were centrifuged at 15,000*g* for 5 min. The supernatant liquid was stored at –20°C.

Following electrophoresis on 5% polyacrylamide gels, the cell lysates were tested for isozymes, esterase, lactate dehydrogenase (LDH), and malate dehydrogenase (MDH). The upper tank buffer consisted of 0.005 *M* Tris and 0.036 *M* glycine (pH 8.3), and the lower tank buffer consisted of 0.0375 *M* Tris (pH 8.9). Vertical slab gels were run at a constant current of 20 *m*A for 3 hr and then stained following the protocol of Harris and Hopkinson (1977).

Restriction and Electrophoresis of DNA

The isolation and purification of polyhedra and virus particles, and DNA preparation of PnNPV from moribund *P. nuda* larvae and infected NTU-PN-HH cells were achieved as described by Summers and Smith (1988). Both DNAs were digested with *Eco*RI, *Hind* III and *Pst*I (Promega Corporation), according to the user's manual, and analyzed in 0.7% agarose gels.

RESULTS

The cell line derived from pupal ovary of *P. nuda* was established *in vitro* and designated NTU-PN-HH. Four major morphologically different cell types (polymorphic, spindle-shaped, round, and squamous) were observed in the primary cell culture. These four cell types were still present after 50 passages, though the polymorphic cells and squamous cells became dominant. The cells have been passaged for more than 4 years in TNM-FH medium containing 10% FBS under a constant temperature of 28°C.

The four major types of NTU-PN-HH cell are easily distinguished after plating for 1 hr. The characteristics of these four cells are as follows (Fig. 1A).

The polymorphic cells (P cells) are irregular in shape and varied in size (20–50 μm wide and 50–90 μm long). The plasma membrane of one side is generally smooth, but the opposite side spreads on the substratum with an irregular margin containing many processes. The nucleus is round or ellipsoid, varied in size (10–25 μm wide and 20–30 μm long), and excentrically located near the smooth margin of the cell.

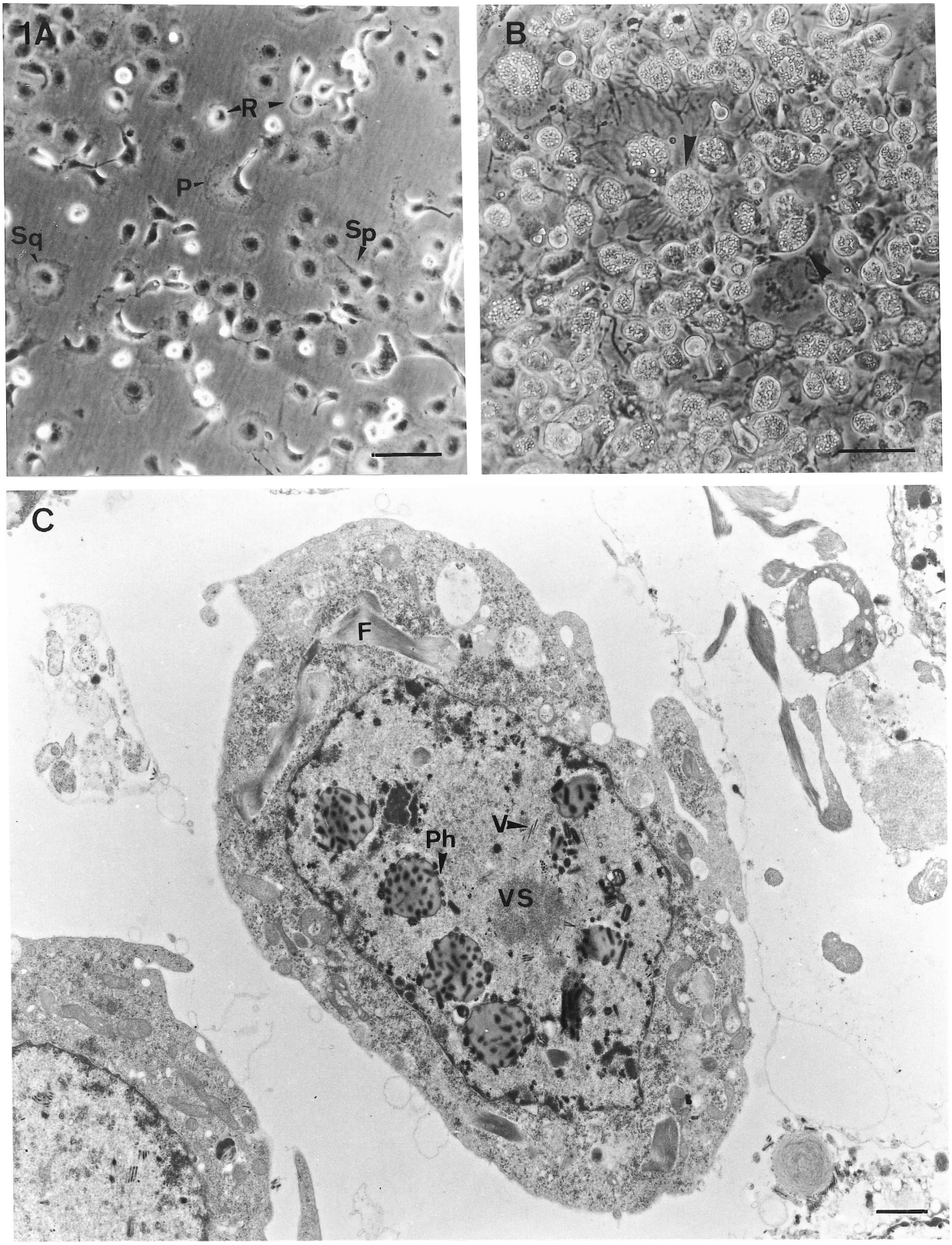

FIG. 1. The characteristic of the NTU-PN-HH cell line established from pupae of *Perina nuda,* showing: (A) Four major cell types in the NTU-PN-HH cell line, polymorphic cells (P), spindle-shaped cells (Sp), squamous cells (Sq), and round cells (R). Bar, 50 μm. (B) The infected cells, 7 days after infection with PnNPV. Note that more than 98% of the cells are susceptible to PnNPV and the hypertrophied nuclei of infected cells fill with polyhedra (arrows). Bar, 50 μm. (C) The electron micrograph of a NTU-PN-HH cell infected with MP strain of PnNPV at 3 days after viral inoculation. Note the virion (V) adjacent to virogenic stroma (VS). The developing polyhedra (Ph) in the hypertrophied nucleus are surrounding virogenic stroma and the fibrillar materials (F) located in cytoplasm and nucleus. Bar, 0.1 μm.

The spindle-shaped cells (Sp cells) are predominantly ellipsoidal, with two extensions (on opposite sides) and varied in size (8–16 μm wide and 65–110 μm). Both or one of the extensions spread on the substratum, with several processes. The cells are able to continue growing on the confluent monolayer, eventually forming a superimposed layer of cells easily detached from the substrate. The nucleus is round or elliptical, centrally located, and almost fills the cell.

The round cells (R cells) are small cells, have a high nucleo-cytoplasmic ratio, and are 20–28 μm in diameter. The nucleus is large, centrally located, and almost fills the cell. The majority of the cells are loosely attached to the substratum, occasionally with one or two tiny filipodia, and the cells form aggregates, especially on confluency.

The squamous cells (Sq cells) are large and varied in size (20–80 μm in diameter). The cells are spreading and adhere firmly to the substrate with processes. The nucleus is round, centrally located, and 13–30 μm in diameter. The cytoplasm may contain granules or vacuoles, especially on confluency.

NTU-PN-HH cells showed a high susceptibility to PnNPV *in vitro* (Fig. 1B). No cytopathic effect was detected in NTU-PN-HH cells after inoculation with HzNPV and BmNPV, whereas a few NTU-PN-HH cells showed a lysis effect after inoculation with AcNPV, especially at lower osmotic pressures (less than 315 milliosmol), but the cells recovered after 3 days of inoculation. The MP strain of PnNPV was isolated *in vitro* and the average yield of PIB per NTU-PN-HH cell was 35. After inoculation with PnNPV MP strain, 98% of NTU-PN-HH cells produced the polyhedral inclusion bodies (PIBs) (Fig. 1B) and the virus titer increased from $TCID_{50/ml} = 10^{-5.61 \pm 0.81}$ to $10^{-8.72 \pm 0.72}$. The morphogenesis and infectivity of PnNPV *in vitro* was similar to that *in vivo.* After 5 days of infection, nucleocapsids, multiple nucleocapsid virions, virogenic stroma, and developing polyhedra were observed in the hypertrophied nuclei, and fibrillar materials were found in the cytoplasm and nucleus. The plasma membrane and nuclear envelope remained intact and showed no sign of destruction. The ultrastructural morphogenesis of PnNPV *in vitro* revealed that PnNPV is a typical NPV (Fig. 1C).

A representative chromosomal spread from NTU-PN-HH cells shows the typical round shape of Lepidopteran chromosome (Fig. 2A). The distribution of chromosome numbers varied widely from 52 to 285, with an average of 117 (Fig. 2B). The influence of various concentrations of FBS on the growth rate of NTU-PN-HH cells at a constant temperature of 28°C indicated that 10 and 20% FBS stimulated cell growth better than did 0 and 5% (Fig. 3A). The doubling times for cell population in TNM-FH medium with 10 and 20% FBS at 28°C were 86 and 54 hr, respectively. The doubling times of NTU-PN-HH cells were also recorded after cultivating the cells in TNM-FH medium supplemented with 10% FBS at different temperatures. The maximum growth rate of the cells was at 28°C (86 hr doubling time). The cells grew very slowly at 4 and 37°C and eventually died, whereas at 20°C, the cells remained viable; cell growth continued slower than at 28°C (94 hr doubling time) (Fig. 3B).

The mobility of esterase, LDH, and MDH from NTU-PN-HH cells was different from that from IPLB-SF-21AE and IPLB-LD-652Y cells (Fig. 4). The restriction endonuclease fragment patterns of the PnNPV DNAs purified from *in vivo* and *in vitro* were identical for *Eco*RI, *Hind*III, and *Pst*I (Fig. 5).

DISCUSSION

The cell line derived from *P. nuda* pupal ovary has been designated NTU-PN-HH. NTU-PN-HH cell line is the second viral-susceptible cell line derived from the

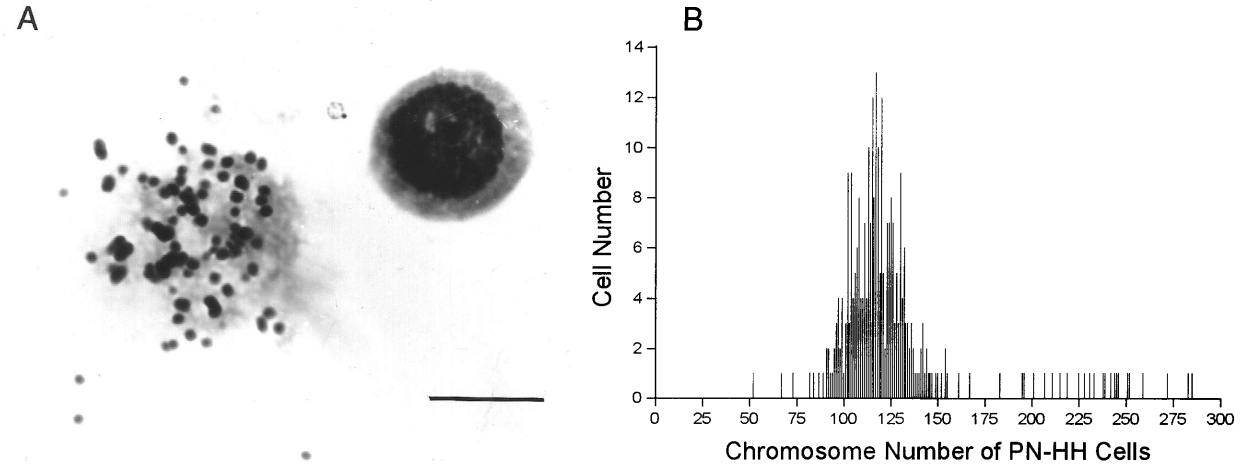

FIG. 2. A representative mitotic chromosomal spread obtained from NTU-PN-HH cells (A) and the distribution of chromosome number in NTU-PN-HH cell population (B). Bar, 10 μm.

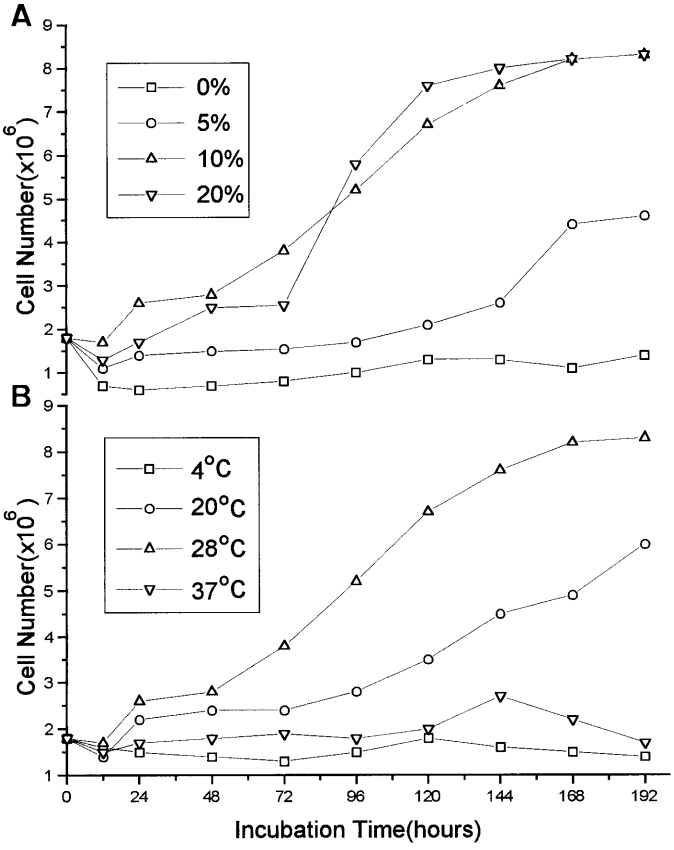

FIG. 3. The influence of cultivation condition on growth rate of NTU-PN-HH cells showing: (A) FBS supplementation (0, 5, 10, and 20%) at 28°C, (B) cultivation temperature (4, 20, 28, and 37°C) with 10% FBS supplement.

species belonging to Lymantriidae (Quiot, 1976; Goodwin *et al.,* 1978). Although an excess of 600 isolates of baculoviruses has been estimated, few of them have been shown to replicate in cultured cells—possibly because investigators have concentrated on viruses and cell lines from insects of economic importance (Kelly, 1982). *P. nuda* is a major garden pest, causing defoliation of *Ficus* spp. The insect is oligophagous, and easy to find from fall to early summer. The serious epizootic disease spread by this insect occurs frequently from late spring. It was considered that the pathogen

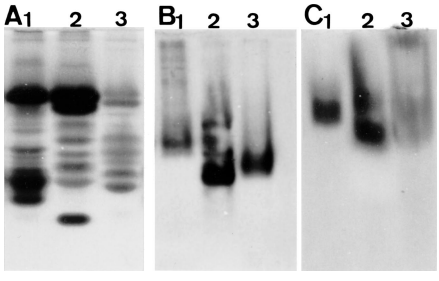

FIG. 4. The isozyme patterns of NTU-PN-HH cells ((A) esterase, (B) LDH, and (C) MDH) (lane 1) compared with IPLB-SF-21AE (lane 2) and IPLB-LD-652Y cells (lane 3).

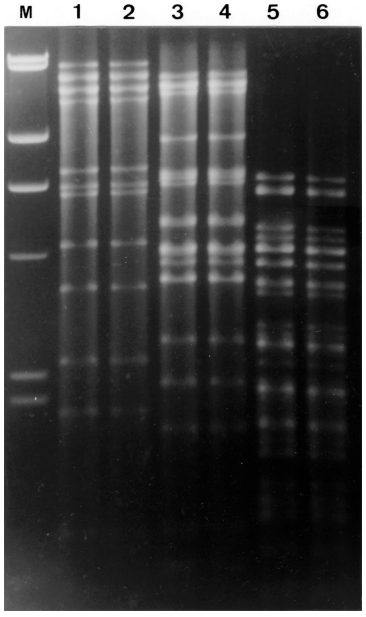

FIG. 5. Restriction endonuclease patterns, *Eco*RI (lanes 1,2), *Hind*III (lanes 3,4) and *Pst*I (lanes 5,6), for PnNPV DNAs purified *in vivo* (lanes 1,3,5) from infected larvae of *Perina nuda* and *in vitro* from infected NTU-PN-HH cells (lanes 2,4,6). M indicates the λDNA/*Hind*III marker.

PnNPV plays an important role in the natural population control (Wang and Tsai, 1995). IPLB-PN-HH cells, because of their high susceptibility to PnNPV, can provide a suitable *in vitro* system for study.

The cell line contains four major morphological cell types, no subclones of NTU-PN-HH were obtained in more than 10 clonal tests. It is believed that there is metabolic coupling among the different morphophic cells. The variability in shape of NTU-PN-HH cells is a result either of the variety of originating cells or the cells being inherently pleiomorphic.

Newly established cell lines should be characterized to distinguish them from all other cell lines, determine the tissue from which the line originated, and assure that is not a contaminant (Hink *et al.,* 1985). Tabachnick and Knudson (1980) demonstrated that 10 isozymes are suitable for distinguishing insect cell lines—three were used in this attempt to identify the NTU-PN-HH cells. Isozyme patterns reveal that the NTU-PN-HH cell line is markedly different from other cell lines that are routinely maintained in our lab.

Several established cell lines failed to replicate their homologous viruses or were semipermissive to the homologous virus (Goodwin *et al.,* 1978; Gelernter and Federici, 1986; Quiot, 1976). The homologous virus, PnNPV, successfully propagated in this newly established cell line (NTU-PN-HH). The four types of NTU-PN-HH cells show similar susceptibility to PnNPV, so the cloned NTU-PN-HH cell is not necessary on the basis of viral susceptibility. Appearance of PnNPV viral progeny in the nucleus and polyhedral formation is

similar to other NPVs (Fraser and Hink, 1982; Hink and Vail, 1973; Potter *et al.,* 1976). NTU-PN-HH cells also presented a problem with respect to the formation of polyhedra. Continued serial passage of PnNPV in this cell line resulted in an increase in the FP (few polyhedra) strain in PnNPV viral progeny. In regard to viral susceptibility of NTU-PN-HH cells, the MP strain of PnNPV within three passages after end-point dilution purification was selected for this assay. In contrast to the wide host range of AcNPV (Danyluk and Maruniak, 1987), PnNPV was found to be host-specific *in vivo* and *in vitro.* It is suggested that the specificity of NPVs is not the result of nutritional factors, but of virus–host relationships concerning receptors or of replication factors (Maeda, 1993) in the virus or cells. The results of comparing the restriction enzyme profiles of PnNPV DNAs purified from two different sources reveals that PnNPV isolated *in vitro* and *in vivo* originated from the same NPV.

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