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A *Perina nuda* cell line (NTU-Pn-HF) from pupal ovary that is persistently infected with a picorna-like virus (PnPV)

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Abstract
A new cell line, designated NTU-Pn-HF, was established from the pupal ovary of *Perina nuda* Fabricius (Lepidoptera: Lymantriidae) and characterized by distinct morphological properties and molecular markers. Pn-HF cells are highly susceptible to *P. nuda* nucleopolyhedrovirus (PenuNPV). Surprisingly, some of the sub-cultural Pn-HF cells suffered an obvious cytopathic effect (CPE) and proceeded to die, while most of the cells remained healthy with good nutrition. Under electron microscopy, most of the Pn-HF cells were found to be carriers of a picorna-like virus. A specific anti-*P. nuda* picorna-like virus (PnPV) antiserum and RT-PCR with a primer set designed from the sequence of a putative PnPV's *helicase* gene confirmed that the picorna-like virus in Pn-HF cells is PnPV (Wang et al., 1999, *J. Invertebr. Pathol*. 74: 62–68), and Pn-HF cells are persistently infected with PnPV. This finding implies that the Pn-HF cell line can be a PnPV supporter for further work, and also a convenient tool in studying the pathogenesis of insect picornavirus and the mechanism of the picornavirus-persistent infection.

Key words: NTU-Pn-HF, PenuNPV, PnPV, persistent infection

INTRODUCTION

The larvae of the ficus transparent wing moth, *Perina nuda* Fabricius (Lepidoptera: Lymantriidae), are external feeders on the foliage of banyan, *Ficus* spp., resulting in tree defoliation in Taiwan and Mainland China. Epizootic diseases of *P. nuda* occur from spring to early summer every year in Taiwan. Two causative agents were identified to be nucelopolyhedrovirus, PenuNPV (Su et al., 1983; Wang and Tsai, 1995) and picorna-like virus, PnPV (Wang et al., 1998, 1999). PnPV has only a low virulence to *P. nuda* larvae, and it has been suggested that PnPV infection has an antagonistic effect on systemic PenuNPV infection in *P. nuda* larvae (Wang et al., 1998). Therefore, the persistent infection of PnPV may play an important enhancing role in maintaining the natural population density of *P. nuda* (Wang et al., 1999).

Drosophila melanogaster cell line can propagate its homologous picorna-like virus (*Drosophila C virus*, DCV) and a heterologous insect picorna-like virus (*Cricket paralysis virus*, CrPV) (Moore and Pullin, 1982; Moore et al., 1981, 1985). The NTU-Pn-HH cell line is another cell line that contributes to the propagation of a picorna-like virus, PnPV (Wang et al., 1996, 1999). In *in vitro* studies, PnPV showed a relatively low cytopathic effect (CPE) on Pn-HH cells, and the obvious CPE of the infected cells occurred at 15 d post-infection. The cytoplasm of the infected Pn-HH cells is filled with round inclusion bodies (IBs) and a few irregular larger IBs are also found (Wang et al., 1999). In the present paper, we describe and characterize a new *P. nuda* cell line, NTU-Pn-HF, which is persistently infected with PnPV. This cell line is also a PenuNPV-permissive cell line. This cell line will facilitate further study on viral persistent infection and viral co-infection.

MATERIALS AND METHODS

Primary culture and subculture. This cell line, designated NTU-Pn-HF, was established from the pupal ovary of *P. nuda* at the same time as establishment of a NTU-Pn-HH cell line (Wang et al.,

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1996). The cells were sub-cultured 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, supplemented with 8% fetal calf serum (FCS) which had been inactivated at 56°C for 30 min. The present Pn-HF cells have been sub-cultured more than 450 passages within 5 y.

Susceptibility of virus. The following two viruses were used to test the viral susceptibility of Pn-HF cells: *Autographa californica* NPV (AcMNPV) and *P. nuda* NPV (PenuNPV), collected from their permissive cell lines, IBL-Sl-1A and Pn-HH cells, respectively (Chou et al., 1996; Wang et al., 1996; Shih et al., 1997). The two permissive cell lines were used as a positive control to test for viral infectivity. The viral infections and the titration of their progenic viruses (AcMNPV and PenuNPV to their susceptible cell lines, Sl-1A and Pn-HH cell lines, respectively) were made as described in a previous paper (Wang et al., 1996).

Chromosome number, growth rate and isozyme analysis. The methods of chromosome number, growth rate of Pn-HF cells and isozyme analysis were as described in a previous paper (Wang et al., 1996).

RAPD-PCR. The confluent Pn-HH and Pn-HF cells were collected and centrifuged at 900 rpm for 10 min at 4°C. Cell pellets were washed twice in phosphate buffered saline (PBS). The extraction of cell DNA was carried out with the QIAamp DNA Mini Kit® (QIAGEN). The random primer kits OPU-A and OPU-BD (20 primers per kit) were purchased from Operon (Alameda, CA) and screened. Two primers were selected: OPU-09 5′-CCA CAT CGG T-3′, and OPU-10 5′-ACC TCG GCA C-3′. PCR was performed exactly as reported in a previous paper (Wang et al., 2000).

Electron microscopy. The Pn-HF cells were harvested after 1 and 5 d incubation by a cell scraper (rubber-policemen) from the flask and centrifuged for 10 min at 900 rpm. The cell pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h at 4°C, and post-fixed in 1% OsO_4 in the same buffer for 2 h at 4°C. After being washed in double distilled water at 4°C, the fixed materials were then dehydrated in an alcohol gradient series and embedded in Spurr Epon. Thin sections were cut with a Reichart OMU 3 ultramicrotome and stained with uranyl acetate and lead citrate. The electron micrographs were taken with a Hitachi H7100 electron microscope operated at an accelerating voltage of 100 kV.

Preparation of rabbit anti-PnPV antiserum. Isolation and purification of PnPV from infected Pn-HH cells were carried out as previously described (Wang et al., 1999). The purified PnPV was mixed with complete Freund's adjuvant (Sigma) for the first inoculum and mixed with incomplete Freund's adjuvant (Sigma) the next three times. A rabbit was injected subcutaneously with antigen emulsion and sacrificed. The serum of the immunized rabbit was prepared as antiserum for PnPV detection.

Indirect immunofluorescent assay. The coverglasses with Pn-HF cells after 1, 5 and 10 d incubation were fixed in cold absolute methanol (4°C) and first reacted with a 1 : 200 dilution of the rabbit anti-PnPV antiserum. These coverglasses were then stained with a 1 : 25 dilution of FITC-labeled goat anti-rabbit IgG $F(ab')_2$ antibody (Sigma), counterstained with 0.05% Evans blue dye (Sigma), and observed under a fluorescence microscope (Olympus BX-50FLA). Staining with contrast Evans blue dye, which fluoresced as a diffuse red background, allowed visualization of both uninfected and infected cell bodies.

Western blotting. Pn-HF cells were prepared as previously described (Tien et al., 1994). Briefly, the cells were lysed with a 1× SDS sample buffer and heated at 100°C for 5 min. Total proteins extracted from the cells were then separated by electrophoresis in a 12.5% SDS polyacrylamide gel, transferred to nitrocellulose paper, and reacted with a 1 : 500 dilution of anti-PnPV antiserum. The immune complexes were further reacted with peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG-HRP, Sigma). Then Opti-4CN™ Substrate Kit (Bio-Rad) was used for color development.

RT-PCR (reverse transcription-PCR). For detecting PnPV in Pn-HF cells, the original, passages number 2, 5, and 10, and new Pn-HF cells grown in a 25 T flask (~3×10^6 cells) were harvested and homogenized in TRIzol® reagent (Gibco BRL), and then subjected to phenol-chloroform extraction and isopropanol precipitation according to the manufacturer's recommendations. The RNA pellet was resuspended in 50 μl DEPC-water and 1 μl was used for the RT-PCR reactions. The RT-PCR reactions were performed by using the Titan™ One

Tube RT-PCR Kit (Roche) and a set of PnPV-specific primers (P51-F3: 5′-ACTAA CGGCC GATCT GTTGG; P179-R4a: 5′-CCATG TTCTG AACCT CTGG), which were designed from the putative *helicase* gene sequence of PnPV genome, and could yield a 610-bp DNA fragment. All reactions were performed according to the protocol provided by the manufacturer. Briefly, cDNA synthesis was at 50°C for 30 min, followed by 35 PCR cycles, each cycle consisting of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and elongation at 68°C for 1 min. There was a final extension step of 7 min at 68°C. Aliquots of 10 μl of amplified products were analyzed in 1% agarose gels in TAE buffer.

RESULTS

Primary culture and subculture

The cell line derived from the pupal ovary of *P. nuda* was established *in vitro* and was designated the NTU-Pn-HF cell line. This cell line has been subcultured in our laboratory for more than 5 y in TNM-FH medium containing 8% FCS at a constant temperature of 28°C. Three major morphologically different cell types, squamous cells (S cells), polymorphic cells (P cells) and round cells (R cells), can be distinguished after 1 h seeding (Fig. 1a). The morphological characters of the three types of Pn-HF cells were similar to their homologous cells, NTU-Pn-HH, but no spindle-shaped Pn-HH cells (SPC) were found in the Pn-HF cells (Wang et al., 1996). The ratio of the three cell types (S : P : R cells) in the Pn-HF cell population is 6 : 3 : 1, but in the Pn-HH cell population (S : P : R : SPC cells) is 5.0 : 2.3 : 2.3 : 0.4. So the S cells are predominant in both cell lines, followed by the P cells. Furthermore, the P cells in the Pn-HF cells can be subdivided into two cell types: large P cells (LP cells) and small P cell (SP cells). The LP cells vary in size, 31–45 μm wide and 70–90 μm long. The eccentric nucleus is located beside the smooth margin of the cells. The SP cells are smaller cells with numerous digitations and come in various sizes, 20–60 μm in diameter. The centric nucleus is round to ellipsoid in shape, the SP cells are seldom found in Pn-HH cells.

After incubation for 3 d, the cells were confluent (Fig. 1b). S cells formed a layer of substratum while R cells and P cells were superposed or located between S cells. The round-shaped vesicles were found in the cytoplasm or beside the nucleus of S and P cells. The large vesicles were in S and LP cells, or free in the medium. Large and small vacuoles were found in the cytoplasm of S and P cells. After 5 d of incubation, the cells overlapped and formed cell aggregations surrounding the margin of S cells. The cells in the cell aggregations contained many vesicles or vacuoles. In longer incubations, some cells became detached and suspended in the medium.

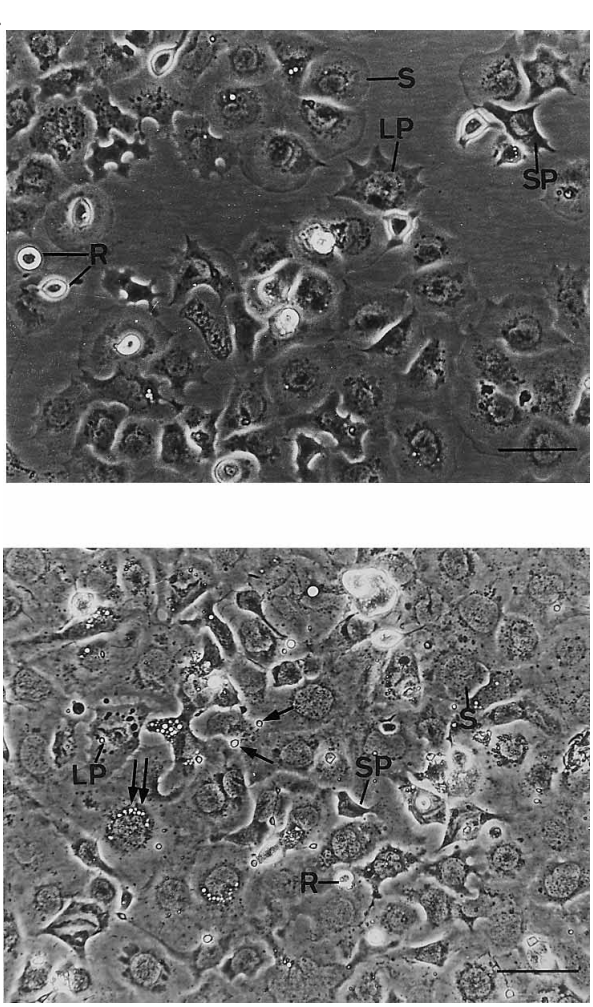

Fig. 1. Micrographs of the cell types of the PN-HF cell line established from pupae of *Perina nuda* showing a: three major cell types, squamous cells (S), large and small polymorphic cells (LP and SP), and round cells (R). b: the confluent cells that contain vesicles near the nucleus or released vesicles (arrows), the vacuoles resulted from the release of inclusion bodies (double arrow). Bar: 50 μm.

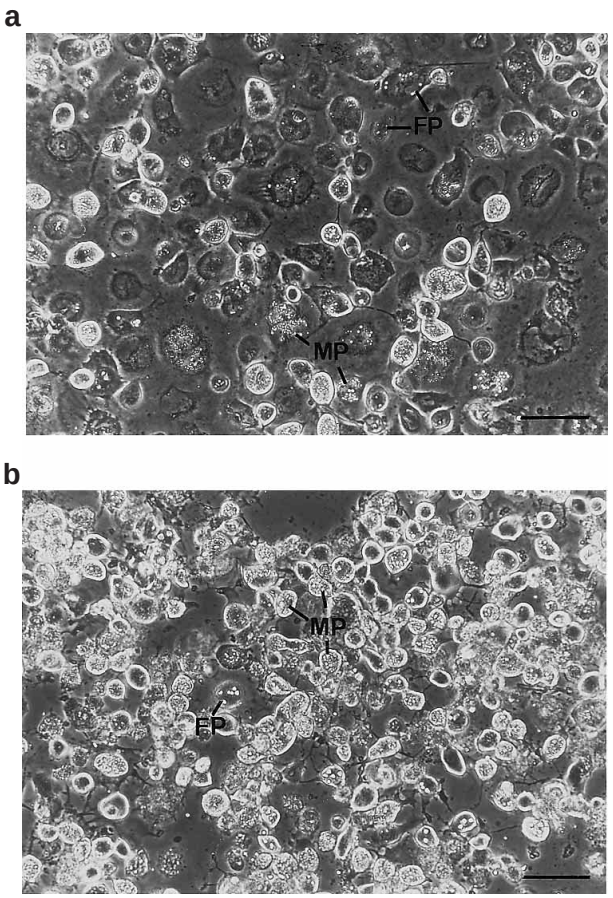

Fig. 2. Micrographs of PN-HF cells (a) and PN-HH cells (b) at 5 d post-infection with PenuNPV. MP: multiple polyhedra; FP: few polyhedra. Bar: 50 μm.

Susceptibility of virus

Pn-HF cells (Fig. 2a) were less susceptible to PenuNPV than Pn-HH cells (Fig. 2b) at 7 d post-infection. As in Pn-HH cells, no CPE occurred in AcMNPV-infected Pn-HF cells. The percentages of occlusion body (OB) contained in the infected Pn-HF and Pn-HH cells with PenuNPV 7 d after inoculation were around 80% and 98%, respectively. The MP (multiple polyhedra, more than 10 OBs per cell) and FP (few polyhedra, less than 10 OBs per cell) of the infected Pn-HF cells were 86% and 14%, respectively, while those of the infected Pn-HH cells were 95% and 5%, respectively. The yields of OB were 1.7±0.1 per Pn-HF cell and 24.1±2.3 per Pn-HH cell. The PenuNPV titers ($TCID_{50}$/ml) 7 d after inoculation in Pn-HF and Pn-HH cells were 3.16×10^6 and 1.05×10^9, respectively.

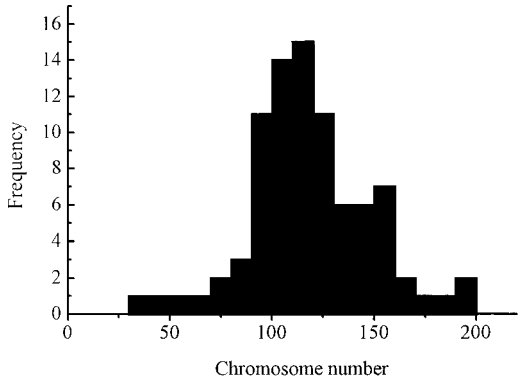

Fig. 3. The distribution of chromosome number in the Pn-HF cell population.

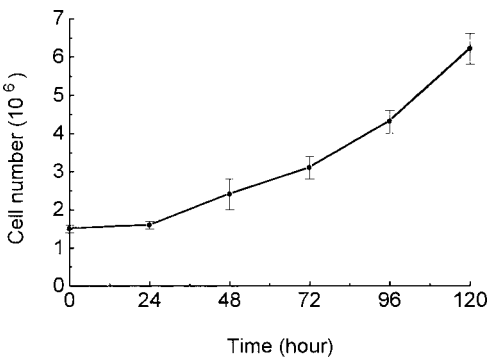

Fig. 4. The growth curve of Pn-HF cells in TNM-FH medium supplemented with 8% FCS at 28°C.

Chromosome number, growth rate, isozyme analysis and RAPD-PCR

Chromosome number

The chromosomal spread obtained from Pn-HF cells showed the typical round shape of the Lepidopteran chromosome, and the distribution of chromosome numbers varied widely from 27 to 200 with an average of 125 (Fig. 3). There was no significant difference from that of Pn-HH cells (52 to 285 with an average of 117).

Growth rate

The growth curve of Pn-HF cells is shown in Fig. 4. The doubling time for the cell population in TNM-FH medium with 8% FCS at 28°C was 52 h (Fig. 4). The doubling time of Pn-HF cells was shorter than that of Pn-HH cells (86 h at 28°C, Wang et al., 1996).

Isozyme analysis

The mobility of MDH and LDH in Pn-HF cells was no different from that of Pn-HH cells (Fig. 5a, b). However, the esterase pattern of Pn-HF cells

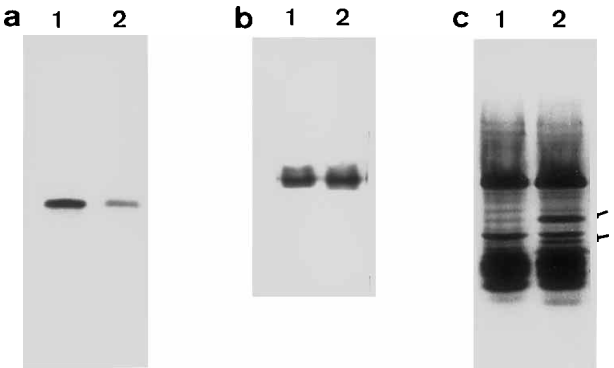

Fig. 5. The MDH (a), LDH (b) and esterase (c) patterns of Pn-HF cells (lane 1) compared with Pn-HH cells (lane 2).

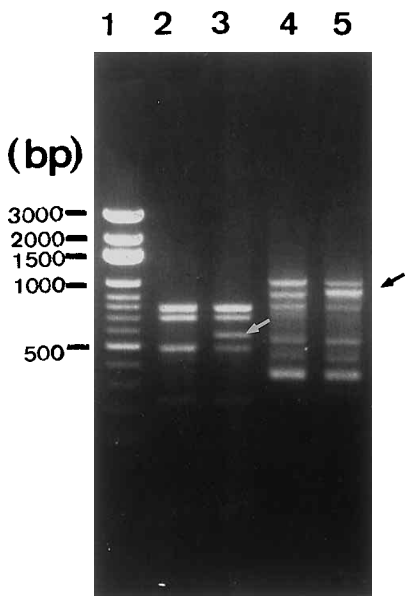

Fig. 6. DNA-based molecular marker for distinguishing Pn-HF cells from Pn-HH cells. The amplicon patterns of the two Pn cells can be distinguished from 500 to 600 bp (white arrow) with OPU-9 primer (lane 2: HF cells; lane 3: HH cells) and 900 to 1,000 bp (black arrow) with OPU-10 primer (lane 4: HF cells; lane 5: HH cells). Lane 1: 100 bp DNA ladder.

could be distinguished from that of Pn-HH cells (Fig. 5c).

RAPD-PCR

In DNA-based markers, the patterns from 500 to 600 bp produced by the OPU-9 primer and from 900 to 1,000 bp by the OPU-10 primer (Fig. 6) could be distinguished by RAPD-PCR assay.

Electron microscopy

Under electron microscopy, the 1-d-old Pn-HF cells showed uniform cell ultrastructure (Fig. 7a). The developing inclusion bodies could be distinguished, and most of them were adjacent to mitochondria in these cells (Fig. 7b). The inclusion-body content of the observed Pn-HF cells was 100%. The developing inclusion bodies contained a few immature viral particles and rod-shaped structures (Fig. 7b). Double membranes surrounded the mature inclusion body, the inner membrane tightly enclosed the inclusion body content (viral particles) and the outer membrane loosely enclosed and formed an irregular intermembrane space. This mature inclusion body occluded viral particles that were either randomly distributed or in a crystalline arrangement, and the isometric-viral particles with the inclusion bodies were about 30 nm in diameter (Fig. 7c). Several cells showed a serious CPE in their hypertrophic cytoplasm. Mitochondria were condensed, vacuoles formed, and endoplasmic reticulum and lysosomes were degenerated with viral particles distributed randomly filling the cytoplasm (Fig. 7d). The percentage of Pn-HF cells with inclusion bodies was up to 100%.

Indirect immunofluorescent assay

Several 1-d-old Pn-HF cells (SP cells) were shown to be seriously infected with virus (a strong PnPV-positive reaction) by immnuofluorescent assay. These cells were recognized by their strong and homogeneous immunofluorescent reaction in the cytoplasm (Fig. 8a). The number of seriously infected cells among 5 and 10-d-old Pn-HF cells increased noticeably, and the cell types were not restricted to SP cells. Small free PnPV-positive particles and cell debris with a strong immunofluorescent reaction were found among the Pn-HF cells (Fig. 8b, c).

Western blotting and RT-PCR

Western blotting

The pattern of Pn-HF cells by Western blotting consisted of four bands, from 31.3 to 24.5 kDa (Fig. 9a). These patterns were similar to that of purified PnPV.

RT-PCR

A predicted 610-bp DNA amplicon was obtained by RT-PCR with a primer set, P51-F3/P179-R4a, and RNAs extracted from Pn-HF cells and purified PnPV (Fig. 9b). The RT-PCR products were further confirmed by sequencing (data not shown). The ex-

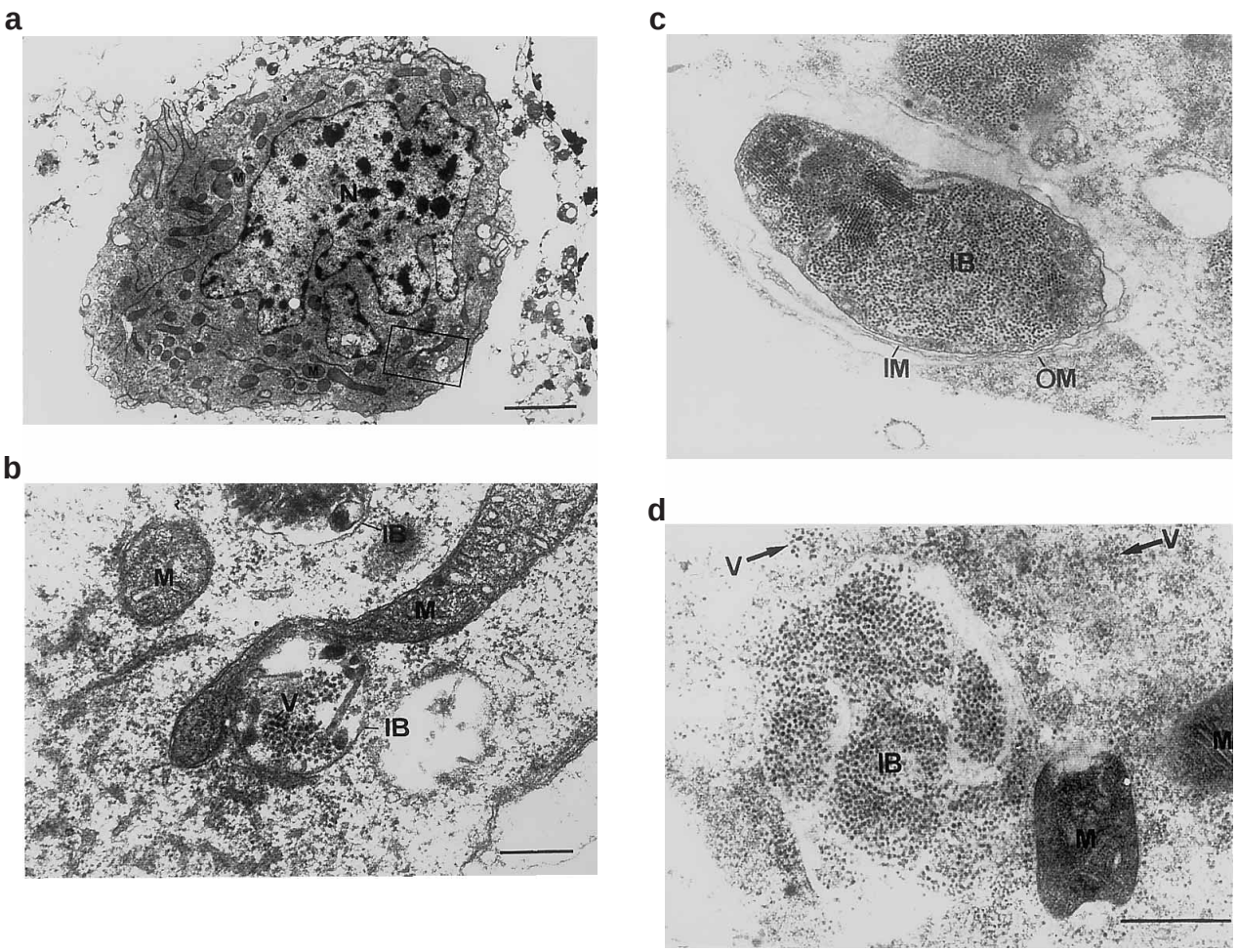

Fig. 7. Electron micrographs of PN-HF cell after 1 d seeding (a, b) and 5 d seeding (c, d). a and b: Note the inclusion bodies (IB) of the 1-d-old Pn-HF cell are adjacent to mitochondria (M), the developing viral particles (V) and the rod-shaped materials are found in the inclusion body. c: The membrane-bound inclusion body (IB) in hypertrophic cytoplasm of a Pn-HF cell contains the mature viral particles distributed in a crystalline arrangement and surrounded with two loose membranes, the viral particles are surrounded tightly by an inner membrane (IM), the outer membrane (OM) is as a barrier for the cytoplasm and IB. d: The viral particles (arrows) are distributed randomly in the cytoplasm of the Pn-HF cell. Bar: a: 2 μm; b, c and d: 400 nm.

amination of PnPV existence in the original, passages number 2, 5 and 10, and new Pn-HF cells by RT-PCR is shown in Fig. 10. These results showed that PnPV existed in Pn-HF cells at the time of establishment and also led us to conclude that the viral particle in Pn-HF cells is PnPV.

DISCUSSION

Based on the characteristics of NTU-Pn-HF cells in morphological properties, chromosome numbers, growth rate, and molecular markers (Figs. 1, 3, 4, 5, 6), Pn-HF is a new cell line, despite sharing many characteristics with its homologous cell line, NTU-Pn-HH (Wang et al., 1996). As its homologous Pn-HH cell line, Pn-HF cells are PenuNPV-susceptible but the virus titer ($TCID_{50}$/ml) and OB yield from PenuNPV-infected Pn-HF cells is much lower than from PenuNPV-infected Pn-HH cells (Wang et al., 1999). Such low performance of PenuNPV in Pn-HF cells suggests that the persistent infection of PnPV in Pn-HF cells interferes with the PenuNPV propagation in Pn-HF cells. In our previous paper, we showed PnPV interferes with OB yield of PenuNPV *in vivo* (Wang et al., 1998). Here we give *in vitro* evidence to confirm our previous conclusion. Although the detailed interference mechanism is still unknown, we hope to

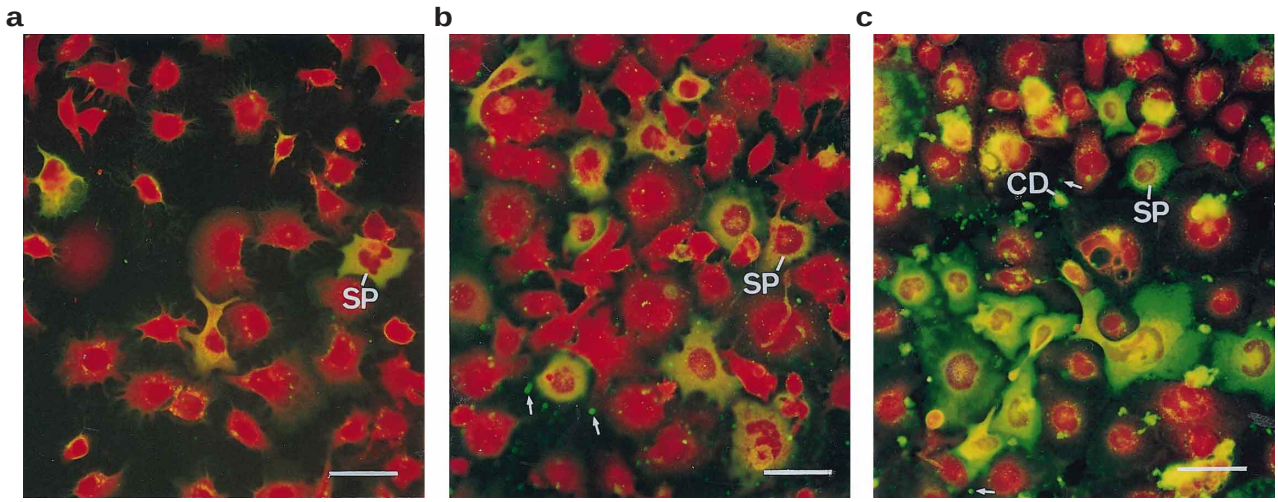

Fig. 8. Evidence of persistent infection with picorna-like virus by indirect immuno-fluorescent assay with rabbit anti-PnPV antiserum. Indirect immunofluorescent assay showed light infection of Pn-HF cell after 1 d seeding (a) while the serious infection of Pn-HF cell after 5 d and 10 d seeding (b, c). Note that only small polymorphic cells (SP) show a strong and homogenous immuno-reaction in their cytoplasm at 1-d-old cells and other cells contain only inclusion bodies in their cytoplasm. In 5- and 10-d-old cells, other cell types show the same reaction too, free inclusion bodies (arrows) and cell debris (CD) with a strong immuno-reaction can be found among the HF cells. Bar: 50 μm.

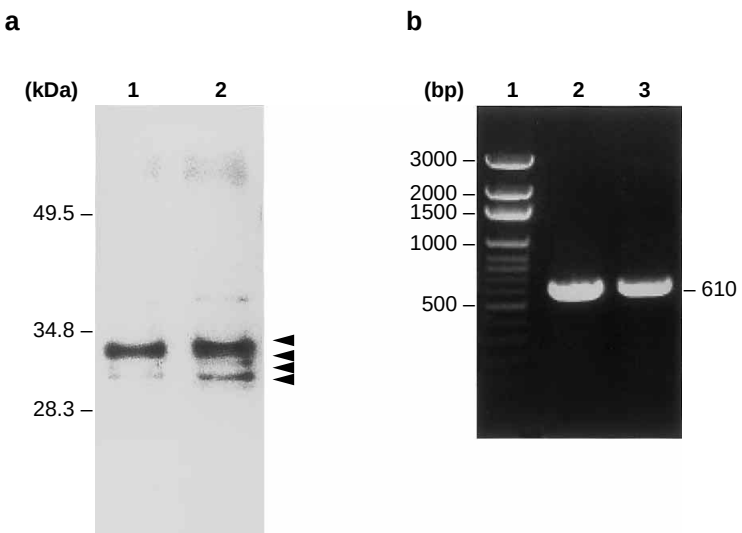

Fig. 9. Detection of PnPV in Pn-HF cells by Western blotting (a) and RT-PCR (b). a: lane 1: Pn-HF cell; lane 2: purified PnPV; b: lane 1: 100-bp DNA ladder; lane 2: Pn-HF cell; lane 3: purified PnPV.

explain it after establishing a titration method for PnPV.

Persistent viral infection occurs commonly in insect populations, especially in laboratory-reared insects, and frequently leads to mass mortality without warning. Persistent viral infection can be converted to productive viral infection by changes in rearing conditions (including temperature, humidity, food quality, and overcrowding) and super-infection with different viruses and/or activators (Tanada and Kaya, 1993). In contrast to lytic viral infections, persistent viral infections can last for a long time without killing the cells. They produce new virus particles by budding out through the cell membranes in a process that causes little damage to the host. In the baculovirus system, persistent infection is caused either by a change in the expression of one cellular genes or more cellular genes

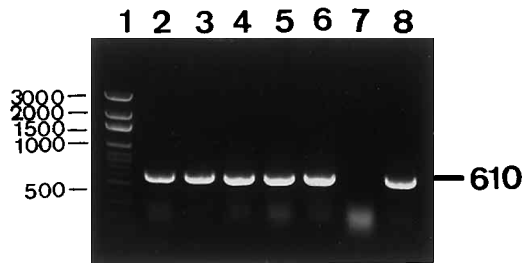

Fig. 10. Detection of persistent infection in Pn-HF cells by RT-PCR. lane 1: 100-bp DNA ladder, lanes 2–6: original, passage number 2, 5, 10, and new Pn-HF cells, lane 7: Sf-9 cells as negative control; lane 8: PnPV genomic RNA.

(Dasgupta et al., 1994), by the transformation of viral genes, e.g. mutated or deleted *p35* gene, or by insertion of an inhibitor of apoptosis gene (*iap*) (Lee et al., 1998). A persistent infection of *Galleria mellonella* cell line virus (GmcIV) in a *G. mellonella* cell line has been reported, and the observable CPE of *G. mellonella* cells can be induced in the presence of the maize stem borer picorna-like virus (MSBV, Lery et al., 1997). Such an evocative phenomenon, i.e.: persistent infection induced into productive infection by other viral infection, was not found in Pn-HF cells, at least, no CPE of Pn-HF cells were observed when the Pn-HF cells were infected with AcMNPV.

In the sub-cultured Pn-HF cells, the ratio of cells with a strong immuno-reaction to anti-PnPV antiserum increased dramatically with longer sub-cultivation (Fig. 8a, b, c). The strong immuno-reaction of the cells implies that the PnPV particles were no longer restricted to inclusion bodies and were distributed throughout the cells, i.e.: these cells suffered a heavy PnPV-infection. Furthermore, the cell debris in the long-term cultured cells (after 5 d sub-cultivation) also showed a strong immuno-reaction, revealing that the heavy-PnPV-infective cells were undergoing death and the lytic process. Based on our study of the PnPV genome (AF323747), we found that there is no gene which has the same function as *p35* in the baculovirus system responding to the persistent infection. Cellular or rearing factors may play an important role in transforming a PnPV-persistent infection to a lytic infection. However, the Pn-HF cell line will provide a convenient tool for understanding the molecular basis of persistent or latent infection *in vitro*. There are few reports about cell lines that are able to support replication of the insect picorna-like viruses. Among them, CrPV and DCV replicate readily in several established *D. melanogaster* cell lines (Moore et al., 1981, 1985; Moore and Pullin, 1982) and PnPV can be propagated in Pn-HH cells (Wang et al., 1999). Now we have a new cell line (Pn-HF) that is persistently infected with PnPV. A comparison of PnPV pathogenesis in both cell lines is a good question to be answered in further studies.

Recently, the genomic analyse of several small insect RNA viruses have been reported and provide a clear picture of the position of classification: for instance, CrPV (Koonin and Gorbalenya, 1992), DCV (Johnson and Christian, 1998), *Plautia stali* intestine virus (PSIV, Sasaki et al., 1998), *Rhopalosiphum padi* virus (RhPV, Moon et al., 1998), and Himetobi P virus (HiPV, Nakashima et al., 1999) have similar genomic organization to that of caliciviruses. These five viruses are recognized as a new genus, the "CrPV-like virus," distinct from the family *Picornaviridae* (van Regenmortel et al., 1999). In contrast, infectious flacheries virus (IFV, Isawa et al., 1998) and Sacbrood virus (SBV, Ghosh et al., 1999) have a genomic organization similar to that of typical picornaviruses (Stanway, 1990). PnPV has a genomic organization similar to that of IFV and SBV (paper in preparation, Genebank accession no. AF323747). Thus, PnPV is closely related to other known members of the *Picornaviridae* and other families within the picornavirus "superfamily." For clarifying the taxonomic position of the insect picorna-like viruses, more studies need to be done, the persistence of PnPV in Pn-HF cells will provide a system for study of PnPV replication and cytopathogenicity and also provide more information about the basic biological aspects of the insect picorna-like viruses.

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