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A Productive Replication of *Hyphantria cunea* Nucleopolyhedrovirus in *Lymantria dispar* Cell Line

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Abstract In this study, comparative replicational properties of *Hyphantria cunea* nucleopolyhedrovirus (HycuNPV) in *Lymantria dispar* (IPLB-LdElta) and *Spodoptera frugiperda* (IPLB-Sf21) cell lines were investigated. Our microscopic observations showed that cytopathic effects (CPEs) in LdElta cells appeared 12 h later than those in Sf21 cells. Whereas polyhedral inclusion bodies (PIBs) formed at 48 h postinfection (p.i.) in LdElta cells, it formed at 36 h p.i. in Sf21 cells. Extracellular virus production determined according to the 50% tissue culture infective dose ($TCID_{50}$) method in LdElta cells started about 12 h later when compared with Sf21 cells. Titers of extracellular virus in LdElta and Sf21 cells were calculated as 1.77×10^9 plaque forming units (PFU)/ml and 5.6×10^9 PFU/ml, respectively, at 72 h p.i. We also showed that viral DNA replication began at 12 h p.i. in both cell lines. Viral protein synthesis was determined by SDS-polyacrylamide gel electrophoresis (PAGE) and polyhedrin synthesis was observed at 12 h p.i. in both cell lines. The results indicate that while the synthesis of macromolecules is 12 h later and production of extracellular virus is almost 3-fold lower in LdElta cells compared with those in Sf21 cells, the LdElta cell line is still a productive cell line for infection of HycuNPV.

Key words: Baculovirus, *Hyphantria cunea* NPV, virus replication, host specificity, LdElta cells

Nucleopolyhedroviruses (NPVs) belonging to the family *Baculoviridae* are large entomoviruses that possess envelope, rod-shaped nucleocapsids containing a double-stranded covalently closed circular DNA genome of approximately 90 to 160 kbp [31]. An understanding of the viral specificity or viral host range of members of the insect virus family *Baculoviridae* is very important, because these viruses have long been considered as potential biological control agents (viral insecticide) of crop and forest pests [12] owing to their relatively high virulence among entomoviruses and their minimal adverse effect on ecosystems and human health. An understanding of the molecular basis of this host specificity is important not only for determining the host range of specific baculoviruses to target particular insect pests but also for determining the safety of the baculoviruses to nontarget organisms.

Nucleopolyhedroviruses have been isolated from more than 520 insect species that span seven different orders [1]. Previous studies that examined cross-infectivity in insect larvae have collectively indicated that each of these NPV isolates exhibited a unique host range that was confined in most cases to a few, if not a single, insect species belonging to the same family [1]. However, some of the NPVs such as *Autographa californica* NPV (AcNPV) and *Anagrapha falcifera* NPV (AfNPV) cause mortality in a broad spectrum of insect species from eight families or more [11]. In addition, studies with cultured cells have demonstrated that NPVs establish unique interactions with different cell lines, resulting in various types of productive and nonproductive infection [15, 21, 22, 34].

Hyphantria cunea NPV (HycuNPV) was isolated and purified from dead larvae near Seoul [6]. The HycuNPVs were used to infect a *Spodoptera frugiperda* IPLB-Sf21 cell line [16, 17]. Little is known about the mechanisms of host specificity of the NPVs and even less is known about the specificity of HycuNPV. Most NPVs are very specific, infecting only members of their host genus. It was shown that the Ld652Y cell line derived from gypsy moth, *Lymantria dispar*, was permissive for *Lymantria dispar* NPV (LdMNPV) [27] and *Orgyia pseudotsugata* NPV (OpMNPV) [3], and semipermissive for AcNPV [24]. However, nothing is known about the susceptibility of gypsy moth cell line, LdElta, infection by HycuNPV. To develop useful host specifity models and to determine the host range of virus, we characterized the extent to which HycuNPV replicates in the *Lymantria dispar* IPLB-LdElta

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cell line and also compared the results with a productive infection of HycuNPV to *Spodoptera frugiperda* IPLB-Sf21.

MATERIALS AND METHODS

Cells and Virus

Insect cell lines used in this study were IPLB-LdElta from *Lymantria dispar* [23] and IPLB-Sf21 from *Spodoptera frugiperda* [30]. LdElta cells were grown at 28°C in unsupplemented ExCell 400 medium (JRH Biosciences) and maintained in 25-cm^2 tissue culture flasks (Grainer) at a 1:6 ratio on a weekly subculture interval. Sf21 cells were also grown at 28°C in TNMFH medium (Grace's Insect Medium) supplemented with 10% heat inactivated fetal bovine serum (Sigma), and maintained in 25-cm^2 tissue culture flasks (Grainer) at a ratio 0.5:5 on a weekly subculture interval [7]. The virus used in these experiments was *Hyphantria cunea* NPV (HycuNPV), obtained from H. H. Lee (Department of Biology, Kon Kuk University, Seoul).

Virus Infection

Cells were seeded at a density of 1×10^6 cell/well to a 6-well culture plate (Grainer), allowed to attach overnight at 28°C, and infected with HycuNPV at a multiplicity of infection (MOI) of 20 PFU per cell. Mock-infected cultures were treated as above, except that the inoculum did not contain any virus. Cytopathic effects were monitored by phase-contrast microscopy (Prior), and photographed at 0, 24, 48, and 72 h p.i.

Virus Titration

Both LdElta and Sf21 cell lines were infected with HycuNPV as described above and incubated at 28°C. The medium from infected cells was harvested by centrifugation at 2,000 $\times g$ for 10 min, and extracellular virus in supernatant was determined by the 50% tissue culture infectious dose ($TICD_{50}$) method of Brown and Faulkner [4] in Sf21 cells. The $TCID_{50}$ titer was calculated by the method of Reed and Muench [26].

DNA Isolation of *Autographa californica* NPV

AcNPV DNA was isolated from extracellular virus produced in AcNPV-infected Sf21 cells. The log-phase Sf21 cells were seeded at a density of 5×10^5 cells/35-mm culture dish (Costar), and cells were infected with AcNPV at MOI of 20 PFU. Viral DNA was isolated from AcNPV-infected Sf21 cells at 3 days p.i. according to the protocol by Invitrogen [13]. Briefly, cells were centrifuged at 5,000 rpm for 3 min, supernatant was transferred into a new microcentrifuge tube, and an equal volume of 20% polyethylene glycol (4°C) was added. After incubation at room temperature for 30 min, the mixture was centrifuged at 13,000 rpm for 10 min. The pellet was suspended in 100 ml of ddH_2O, and 10 ml of proteinase K (10 mg/ml, Sigma) was added, and incubated at 50°C for 1 h. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) was added and the mixture centrifuged at 13,000 rpm for 10 min. The upper phase was taken, and to which sodium acetate, glycogen, and 100% ethanol were added. After incubation to precipitate DNA at -20°C for 20 min, the mixture was centrifuged at 13,000 rpm at 4°C for 10 min. Finally, the pellet was washed with 70% ethanol, resuspended in 10 ml of ddH_2O, and stored at 4°C.

Probe Preparation

A fragment of AcNPV DNA was amplified, using specific primers for the *polh* gene [forward (F): 5' TAC GTG TAC GAC AAC AAG T-3' and reverse (R): 5' TTG TAG AAG TTC TCC CAG AT-3'] [10] in a total volume of 50 µl, by polymerase chain reaction (PCR) in a thermal cycler (Hybaid PCR Sprint Thermal Cycler) [33]. The reaction mixture included 4 µl of AcNPV DNA, 5 µl (10×) PCR buffer, 1 µl (10 mM) dNTP mix, 2 µl F and R primer DNAs (10 µM), 3 µl $MgCl_2$ (25 mM), and 0.5 µl Taq DNA polymerase (2.5 U). The cycling parameters for PCR were as follows: after an initial denaturation step for 2 min at 94°C, the reactions were carried out successfully under 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C for 30 cycles, and finally 7 min at 72°C. Then, the PCR product was electrophoresed on a 1% agarose gel, and the DNA fragment was cleaned from the gel by using the Concert Gel Extraction System (Gibco BRL). DNA fragments were then labeled with DIG and the labeled probe was stored at -20°C until used.

Preparation of Samples for Slot Blot Hybridization

LdElta and Sf21cells were seeded at a density of 1×10^6 cells/well to a 6-well culture plate (Grainer), and infected with HycuNPV at MOI of 20 PFU per cell. Culture medium was removed at 0, 6, 12, 24, 48 h p.i. and cells were lysed by adding 800 ml of 0.5 N NaOH into each well [8]. The suspension was neutralized with 80 ml of 10 M NH_4-acetate and was stored at 4°C.

The immobilon transfer membrane (Millipore) was used for hybridizations and 100 ml of cell lysate for each time was applied to the membrane under vacuum. The prehybridization and hybridization were carried out by using the Dig High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics) as described by manufacturer.

Detection of Infected Cell-Specific Protein (ICSP) Synthesis

LdElta and Sf21cells in a 6-well plate (1×10^6 cells/well) were infected with HycuNPV at MOI of 20 PFU. The infected and mock-infected cells were harvested at 0, 24, 48, and 72 h p.i. by centrifugation at 5,000 rpm for 10 min

at 4°C and lysed by a lysis buffer (0.1% Triton X-100 including 50 μg/ml leupeptin and 100 μg/ml pepstatin A, prepared in PBS) [13]. Protein extraction procedures were followed as described by O'Reilly *et al.* [25]. Proteins were electrophoresed by 10% SDS-PAGE at 30 mA according to Laemmli [14], and stained with Coomassie brillant blue R-250 as described by Sambrook *et al.* [28].

RESULTS

Cytopathic Effects and Polyhedral Inclusion Body Formation

To determine if the cell lines differed in the susceptibility to HycuNPV, LdElta and Sf21 cells were monitored for cytopathic effects (CPEs) and polyhedral inclusion body (PIB) formation. The mock-infected cells appeared normal throughout the incubation period. Infection of the Sf21 cell line with HycuNPV showed pronounced CPEs in which the nuclei of the cells were a little swollen and the nuclear membranes were hypertrophied to the cell membranes, by 24 h p.i. At 36 h p.i., PIB formation was observed in the Sf21 cells (Fig. 1), and approximately 60–65% of total cells had numerous PIBs. Although cells were generally granulated and rounded, and developed nuclear hypertrophy, some cells lost general shape by extending. At 48 h p.i., cells began to darken, and 95% of the cells had PIBs. At 72 h p.i., all the infected cells contained many polyhedra, and some cells were beginning to detach from the bottom of the flasks. As the cells became more hypertrophied and lyzed, PIBs in nuclei released into the culture media.

When the LdElta cell line was infected with HycuNPV, the infection process was slower than that in the Sf21 cells. CPEs such as granulated, swollen, and nuclear hypertrophy appeared at 36 h p.i. and PIB was first observed at 48 h p.i. (Fig. 1). By 72 h p.i., although 90% of LdElta cells contained polyhedra, no cells were detected lyzed. Only very few cells detached from the matrix.

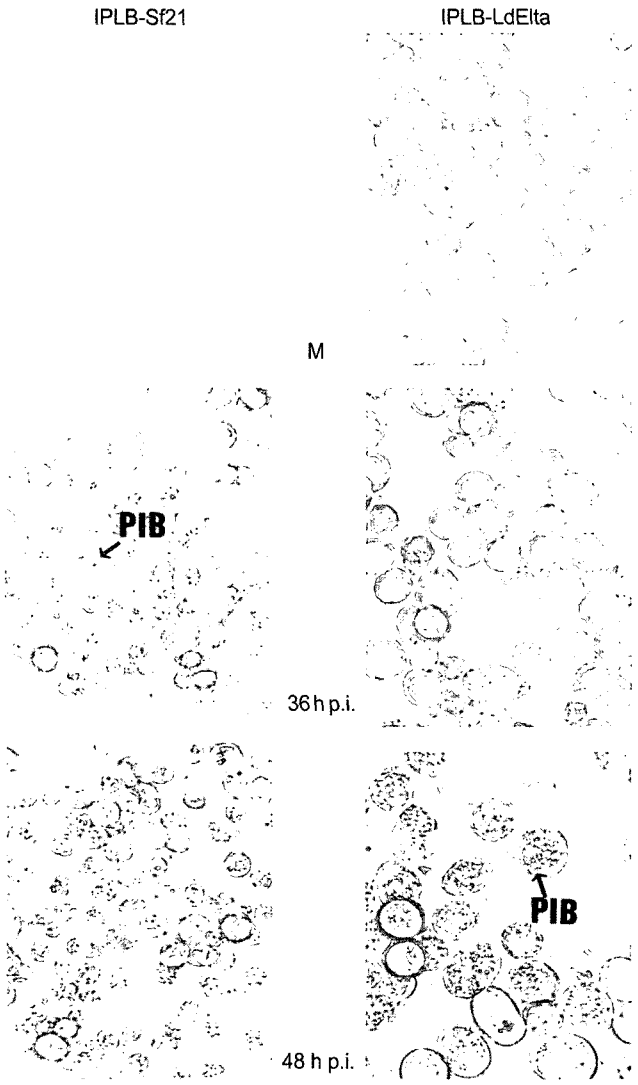

Fig. 1. Microscopic examination of IPLB-Sf21 and IPLB-LdElta cell lines infected with HycuNPV at input MOI of 20 PFU/ml.
M, Mock-infected cells; PIB, Polyhedral inclusion body. Cytopathic effects and PIBs formation in Ld cells appeared 12 h later than those in Sf21 cells.

Infectious Virus Production in LdElta and Sf21 Cell Lines Infected with HycuNPV

Culture media were harvested from the HycuNPV-infected cells at 0, 24, 48, and 72 h p.i. and the yield of budded virion (BV) in the culture media was titered by the 50% tissue culture infectious dose ($TCID_{50}$) method using the Sf21 cell line. The results showed that HycuNPV replicated in both Sf21 and LdElta cell lines and released BVs at different amounts into the culture medium (Table 1). The infectious virus yield at 24 h p.i. in Sf21 was 3.87×10^8 PFU/ml, which represented a 10^4-fold increase in titer. The number of BVs in the other samples were 1.6×10^9 PFU/ml and 5.6×10^9 PFU/ml at 48 and 72 h p.i., respectively. On the other hand, in the LdElta cell line, infectious viruses were yielded as 1.42×10^4 PFU/ml, 5.6×10^8 PFU/ml, and 1.77×10^9 PFU/ml at 24, 48, and 72 h p.i., respectively.

Viral DNA Replication

To compare HycuNPV DNA replication in the Sf21 and LdElta cell lines, viral DNA levels in infected cells were

Table 1. Endpoint dilution titers in HycuNPV-infected Sf21 and LdElta cell lines.

Cell lines	Titers[a] (PFU/ml)			
	0 h p.i.	24 h p.i.	48 h p.i.	72 h p.i.
Sf21	4.32×10^4	3.97×10^8	1.6×10^9	5.6×10^9
LdElta	1.42×10^4	5.6×10^4	5.6×10^8	1.77×10^9

[a]Virus titers were determined by the 50% tissue culture infectious dose ($TCID_{50}$) method, as described under Materials and Methods.

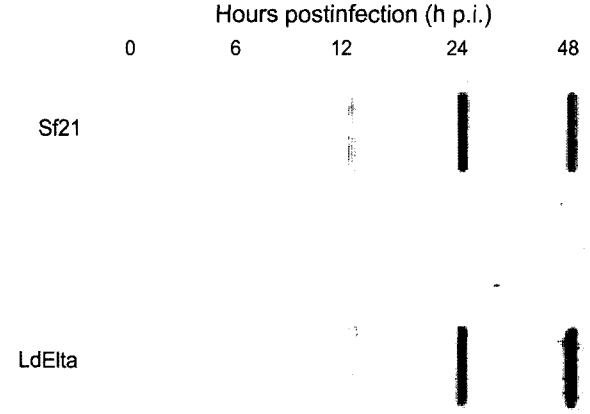

Fig. 2. Slot-blot analysis of viral DNA in Sf21 and LdElta cells infected with HycuNPV.
At 0, 6, 12, 24, and 48 h p.i., infected cells were harvested and viral DNA was detected by using DIG kit. The viral DNAs were blotted onto transfer membrane and hybridized with labeled AcMNPV DNA (prob) as described under Materials and Methods.

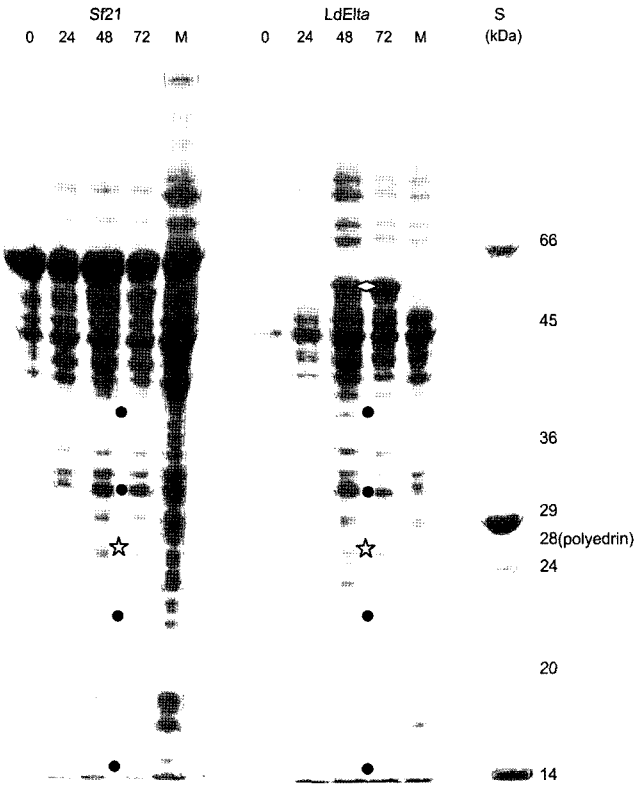

Fig. 3. SDS-PAGE analysis of viral structural polypeptides and polyhedrin in Sf21 and LdElta cells.
Cells infected with HycuNPV were harvested at 0, 24, 48, and 72 h p.i., and lyzed. Polypeptides from the lyzed cells were resolved on 12% SDS-polyacrylamide gel and stained with Coomassie brillent blue. M: Mock-infected cells; S: Standart molecular masses; (☆) Polyhedrin protein; (●) Others viral proteins in both cell lines; (◇) Viral protein in LdElta cell line only.

investigated by slot blot hybridization. Viral DNA was extracted from infected cells at 0, 6, 12, 24, and 48 h p.i., blotted onto a transfer membrane, and hybridized with labeled AcNPV DNA, as described under Materials and Methods. In HycuNPV-infected Sf21 and LdElta cells, viral DNA replication was initiated by 12 h p.i. and reached at high levels by 24 h p.i. The rate of synthesis was maximal between 24 and 48 h p.i. (Fig. 2).

Viral Protein Synthesis

Experiments were conducted to examine if infected cell-specific proteins (ICSPs) were synthesized in HycuNPV-infected Sf21 and LdElta cell lines. At 0, 24, 48, and 72 h p.i., lysates from the infected cells were electrophoresed on SDS-polyacrylamide gels, and protein bands were visualized by staining with Coomassie brillant blue. Polyhedrin, the major NPV structural protein, expressed at molecular mass of 28 kDa in both cells at 24 h p.i. (Fig. 3). It was observed as a dense band at 48 and 72 h p.i. At 48 and 72 h p.i., other ICSPs, with molecular masses of approximately 37 kDa, 30 kDa, 23 kDa, and 14 kDa, were detected. In addition, another protein with an approximate molecular mass of 55 kDa was also observed in only the LdElta cell line at 48 and 72 h p.i.

DISCUSSION

The susceptibility of *Lymantria dispar* cell line, LdElta, to HycuNPV was studied on the basis of CPE, PIB formation, BV production, viral DNA replication, and viral structural protein synthesis. The results were compared with Sf21 infected with HycuNPV. Although CPE manufacture and PIB formation were delayed by 12 h in LdElta cells, both Sf21 and LdElta cell lines were highly susceptible to HycuNPV and displayed characteristics typical of productive infections, including granulation, swelling, nuclear hypertrophy, the presence of inclusion bodies, and nuclear darkening (Fig. 1) [2, 32]. At 72 h p.i., all of the Sf21 cells and 90% of LdElta cells had PIBs. These results are similar to those of other NPV replication studies [5, 9, 18].

The results of CPEs were supported by the infectious virus yields from the Sf21 and LdElta cell lines. The basal viral titers were approximately 1×10^4 PFU/ml in both cell lines. Whereas the greatest increase in the amount of BV present in Sf21 cells occurred at 24 h p.i., that in LdElta cells occured at 48 h p.i. The increase in the amount of BVs in both cell lines appears to be consistent with the observation of CPEs and PIBs. McClintock *et al.* [24] have shown that a gypsy moth cell line was semipermissive for AcNPV. Our observations showed that the gypsy moth cell line is more sensitive to HycuNPV than AcNPV (data not shown). Riegel and Slavicek [27] determined that BV from LdNPV-infected Ld652Y cells was released between 24 and

36 h p.i., and polyhedra were first detected at approximately 48 h p.i. We demonstrated that the LdElta cell line was permissive for HycuNPV, and BV yield and PIB formation were similar to the results of Riegel and Slavick. In a previous study, Shirata *et al.* [29] reported that HycuNPV-infected Sf21 cells yielded BVs into the culture medium but were defective in polyhedrin expression and PIB formation. In addition, in this study, we also observed that both polyhedrin was expressed and PIB formed in HycuNPV-infected Sf21 cells (Figs. 1 and 3), as previously reported by Lee [16].

Slot blot hybridization data showed that DNA replicated efficiently in both cell lines at 12 h p.i., and the maximal rate of viral DNA synthesis was between 24 to 48 h p.i. A previous study reported that AcNPV DNA synthesis in Ld652Y cells, a semipermissive gypsy moth cell line for AcNPV, was initiated from 8 to 12 h p.i., continued by increasing rate from 12 to 20 h p.i., and declined from 20 to 36 h p.i. [24]. Another study also indicated that LdNPV DNA replication in the same cells started at approximately 18–20 h p.i. [27]. HycuNPV exhibited a very similar DNA replication time course in both Sf21 and LdElta cells as determined from slot blot hybridization (Fig. 2). The previously reported results, comparable to our results, determined that the HycuNPV-LdElta cell system was more productive than LdNPV-Ld652Y and another NPV-gypsy moth cell system.

The results of SDS-PAGE showed that polyhedrin protein synthesis occurred in HycuNPV-infected Sf21 and LdElta cells at 24 h p.i., and reached at higher levels at 48 h p.i. and 72 h p.i., respectively. Polyhedrin is the product from a very late gene and its expression is dependent upon the transactivation by the products from the very late expression factor-1 gene and some of the late expression factor genes including *ie-1* and *lef-2* [20]. With the exception of polyhedrin, various viral proteins synthesized also in both cell lines at 48 h p.i. These results are similar to a productive infection study reported by Liu and Bilimoria [19].

Successful NPV replication leading to productive infection proceeds through several sequential steps, which include entry of the virions into host cells, early viral gene expression, genomic DNA replication, late viral gene expression, assembly and release of BVs, very late gene expression, and crystallization of polyhedrin into polyhedra that occlude virions with a phenotype different from that of BVs, as understood in this study. Such an NPV replication cycle may abort at any step after entry into the host cells.

This study indicated that infection of IPLB-LdElta cells by HycuNPV was successfully completed. Therefore, Ld-HycuNPV infection is productive. However, the results indicate that the synthesis of macromolecules is accomplished 12 h late and production of extracellular virus is almost 3-fold lower in LdElta cells compared with Sf21 cells. This might be due to incompatibility of some of the host transcriptional factors or deficiency of some virus transactivation factors in Ld-HycuNPV replication.

Thus, the present study developed an NPV-cell system that exhibited distinct interactions between NPV and host cell. This NPV-cell system developed in the present study should lead to an understanding of the cellular and viral factors critical for defining the host specificity of virus gene expression.

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