

New Cell Lines from *Heliothis virescens*: Characterization and Susceptibility to Baculoviruses

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New cell lines from embryos of *Heliothis virescens* were recently developed. Six primary cultures were initiated in June 1995. From these initial cultures, two produced sufficient cell growth to allow subcultivation and eventually led to the establishment of seven cell strains, three of which are maintained at low temperatures (17°C). The strains were compared with a previously established cell line from *H. virescens* by isozyme analysis and shown to be from the same species. All the strains were inoculated with various baculoviruses, including *Autographa californica* nucleopolyhedrovirus (NPV), *Anagrapha falcifera* NPV, *Anticarsa gemmatalis* NPV, *Rachoplusia ou* NPV, *Lymantria dispar* NPV (LdMNPV), *Orgyia pseudotsugata* NPV (OpSNPV), *O. leucostigma* NPV (OIMNPV), and *Helicoverpa zea* NPV (HzSNPV). All seven strains were highly susceptible to the noctuid NPVs, and large numbers of occlusion bodies (OBs) were produced in most of the inoculated cells. The HzSNPV infection developed at a slower rate (requiring 1 week or more before a substantial number of cells contained OBs compared with 2–3 days for the other three noctuid NPVs). Three of the *H. virescens* strains were also susceptible to OpSNPV although only 10–20% of the cells produced OBs with this virus. We did not observe cytopathology (CPE) in any cells inoculated with OIMNPV or LdMNPV. Our results suggest that these new strains can be useful for the study and possibly the production of baculoviruses for which no effective cell systems are available and for comparative studies on multiple virus species.

Key Words: *Heliothis virescens* cell lines; Baculoviridae; *Autographa californica* nucleopolyhedrovirus; *Anagrapha falcifera* nucleopolyhedrovirus; *Anticarsa gemmatalis* nucleopolyhedrovirus; *Rachoplusia ou* nucleopolyhedrovirus; *Orgyia pseudotsugata* nucleopolyhedrovirus; and *Helicoverpa zea* nucleopolyhedrovirus.

INTRODUCTION

The tobacco budworm, *Heliothis virescens*, is part of a complex of related species in the *Heliothis/Helicoverpa*

genera that are very serious pests of cotton, corn, tomato, and tobacco. This insect quickly develops resistance to chemical pesticides and is a prime target for biological controls, including insect viruses (Ignoffo, 1966). While other cell lines have been established from this insect (McIntosh and Ignoffo, 1983; Lynn *et al.*, 1988), the yields of baculovirus occlusion bodies (OBs) in these cells were lower than can be obtained in other lepidopteran lines (McIntosh *et al.*, 1985). All of these lines were established from larval or pupal reproductive tissue. In previous studies with cell lines from the gypsy moth, we have shown other cell sources for established cell lines, including embryos, can provide better substrates for the replication of nucleopolyhedrovirus (NPV) (Lynn *et al.*, 1989).

In this study, we used *H. virescens* embryos to initiate cultures. Of six initial primary cultures, two showed sufficient cell growth to enable subculturing and led to the development of cell lines. Seven different strains were established as a result of various selection and growth conditions. These strains were characterized by morphology and by isozyme analyses. They were subsequently screened for replication of various NPVs and shown to be susceptible to *Autographa californica* NPV (AcMNPV), *Anagrapha falcifera* NPV (AfMNPV), *Anticarsa gemmatalis* NPV (AgMNPV), *Rachoplusia ou* NPV (RoMNPV), *Orgyia pseudotsugata* NPV (OpSNPV), and *Helicoverpa zea* NPV (HzSNPV). These new lines should be useful in the study and production of baculoviruses, especially HzSNPV for which no satisfactory system is currently available.

METHODS AND MATERIALS

Cell line development. Primary cultures were isolated from middevelopment *H. virescens* eggs obtained from Dr. James Foster (Dupont Ag, Newark, DE) using procedures described in Lynn (1996). Six primary cultures were initiated in BML-TC/10 medium (TC-100 from Sigma Chemical Co., St. Louis, MO) modified as previously described (Lynn *et al.*, 1988) in June, 1995 and maintained at 26°C. Medium was added or re-

placed on these cultures at 7- to 14-day intervals until enough cells were available for subculturing. Of the initial six cultures, two developed into cell lines (initially designated IPLB-HvE1 and IPLB-HvE6).

During early passages, the HvE6 line showed heterogeneous morphologies between the attached and suspended cell types. Two separate strains were isolated from this culture by selecting for attachment and nonattachment, respectively, and were designated as IPLB-HvE6a and IPLB-HvE6s. The attached strain was selected by firmly striking the culture prior to replacing the medium at weekly intervals (or at the time of subcultivation when cell growth was sufficient to actually split the cultures). For the suspended strain, cultures were treated gently to avoid disturbing attached cells when transferring cells to a new culture. After 9 months in culture (4 to 15 passages), we began to maintain separate cultures of the three strains at 17°C in addition to the 26°C standard temperature (these low temperature strains were designated IPLB-HvE1-lt, IPLB-HvE6a-lt, and IPLB-HvE6s-lt). An additional suspended substrain was isolated from HvE1 (now designated IPLB-HvE1a and IPLB-HvE1s) resulting in a total of seven strains.

Maintenance. Because of differences in growth characteristics between the attached and suspended strains, different cell passage procedures are necessary. The four attached strains (HvE1a, HvE1-lt, HvE6a, and HvE6a-lt) were subcultured using trypsinization. The medium was decanted from a confluent 25-cm² culture and the cell monolayer was rinsed with 2.0 ml trypsinization buffer (Ca- and Mg-free Hanks' phosphate-buffered saline; Lynn, 1996). The rinse was discarded and an additional 1.0 ml of buffer containing 50 µg/ml VMF trypsin (Worthington Enzymes, Freehold, NJ) was added. After 5 min at room temperature, the trypsin solution was decanted and the culture held for an additional 5–10 min. Fresh TC-100 medium was then added and the cell suspension was distributed to new cultures.

For the suspended cell strains (HvE1s, HvE6s, and HvE6s-lt), medium from a mature culture was simply transferred to fresh TC-100 medium in a new flask. If the split ratio was less than 1:4, the cell suspension from the mature culture was centrifuged (5 min at 50g) and resuspended in fresh medium before transfer to the new culture flask.

Since these cultures were initiated, the cells have been maintained in Falcon, Nunclon, or Greiner culture flasks with no discernible difference in growth. In the early stages of establishment, all cultures were split at 1:2 after cultures reached sufficient density as judged by microscopic examination. The split ratios were altered as the cell growth rate increased during adaptation to the culture conditions. The 26°C cultures are currently subcultured on a 1-week schedule and the 17°C cultures are subcultured at 2-week intervals.

Characterization. Extracts of the cells from the seven strains were prepared using the Authentikit isozyme system. The extracts were applied to 1% agarose gels, electrophoresed at 160 V for 25 min, and then stained for malic enzyme (ME, EC 1.1.1.40), isocitrate dehydrogenase (ICD, EC 1.1.1.42), phosphoglucose isomerase (PGI, EC 5.3.1.9), or phosphoglucumutase (PGM, EC 2.7.5.1). The stains, buffers, and gels were obtained from Innovative Chemistry (Marshfield, MA). An extract from IPLB-HvT1 cells (Lynn *et al.*, 1988) was included on the gels for comparison and the isozyme migration patterns were also compared with other cells maintained in our laboratory.

Virus studies. The viruses tested in these studies include AcMNPV, AfMNPV, AgMNPV, RoMNPV, OpSNPV, *O. leucostigma* NPV (OIMNPV), *Lymantria dispar* NPV (LdMNPV), and HzSNPV. The virus inocula were all derived from cell cultures that are permissive for the respective viruses. The source of viruses was described previously, Lynn and Shapiro (1997), except for HzSNPV. This virus was prepared by per os infection of third instar *H. zea* larvae on an artificial diet (Bell *et al.*, 1981). Infected larvae were surface disinfected by immersion in 70% ethanol for 10 min, air dried, and then bled through a cut proleg into cold TC-100 medium (on ice) supplemented with 50 µg/ml gentamicin sulfate (Sigma) and 80 µg/ml reduced glutathione (Sigma). One milliliter of medium was used per larvae (infected larvae were approximately 2.5–3 cm long). The hemolymph-containing medium was centrifuged (15 min at 350g) and the supernatant was passed through a 0.45 µm Uniflo filter. This filtrate was used to initiate infection of IPLB-HvT1 cells and supernatants from infected cultures were used for subsequent tests.

To screen for virus susceptibility, cells of each strain were transferred to 24-well tissue culture plates (Costar) in 1.0 ml TC-100 medium supplemented with 50 µg/ml gentamicin sulfate. Ten to 100 µl of each virus inocula was added to a well of each cell strain. Due to the growth characteristics of some of the *H. virescens* cell strains, accurate cell counts were not possible. However, based on microscopic examination of the cultures, we estimated that the virus inocula used in these studies represent a multiplicity of infection of 0.1–10 infectious particles per cell (as determined by endpoint assay of each virus in an appropriate cell line). In our experience, this level of inoculation should be adequate for determining susceptibility of these cells to the various viruses. The inoculated plates were sealed with masking tape and maintained at room temperature (approximately 22°C) and examined with an inverted phase contrast Leitz microscope at intervals up to 1 month for the presence of viral OBs.

A quantitative study was performed on the new strains with HzSNPV since no good cell systems exist for its study or production. Cells were inoculated into

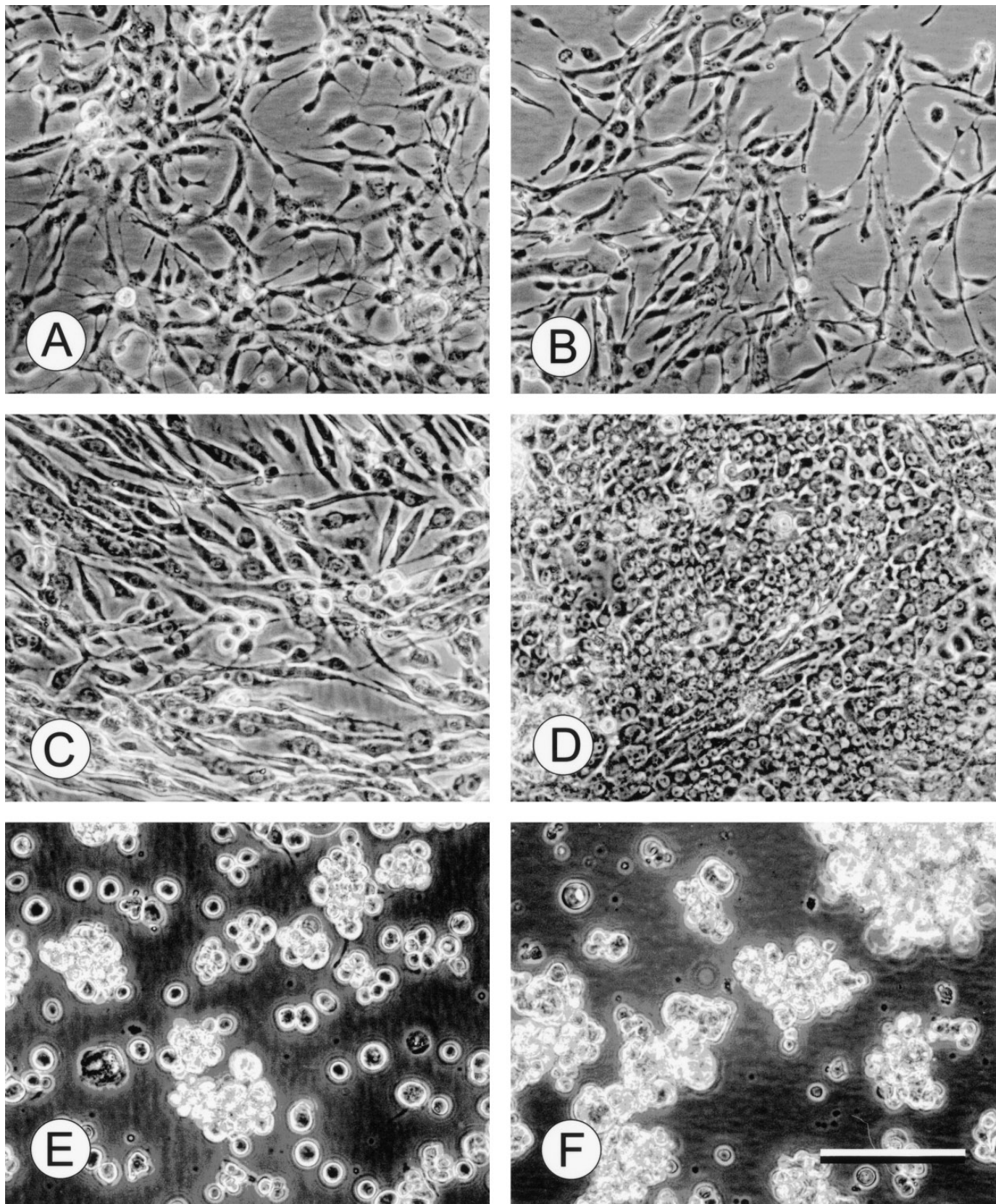


FIG. 1. . Phase contrast micrographs of new *H. virescens* cell strains. (A) IPLB-HvE1a; (B) IPLB-HvE1-lt; (C) IPLB-HvE6a; (D) IPLB-HvE6a-lt; (E) IPLB-HvE1-s; (F) IPLB-HvE6s. All figures are at the same magnification. Bar, 100 μ m.

24-well culture plates as above. Cultures were initiated at three densities equivalent to 0.5, 1.0, and 2.0 \times the density routinely used to initiate cultures during normal cell passage (again, this method was used because of the inability to make accurate counts of the aggregated cells in some of the cultures). The cells were inoculated with 10 μ l HzSNPV from previously infected cell cultures (two passages in HVE6a cells). Plates were sealed with masking tape and maintained at room temperature for 9 days. The medium was replaced with 1 ml deionized water and 40 μ l 10%(w/v) sodium laurel sulfate (Sigma) was added. Repeated pipetting through a glass Pasteur pipette (Corning Glass Works, Corning, NY) was used to disrupt the cells. The plates were set aside for 1–3 days to allow the OBs to settle, after which an estimate of OBs was determined by counting a specific surface area in each well as measured by a photographic ocular in a Leitz inverted phase contrast microscope.

RESULTS AND DISCUSSION

Seven cell strains have been established from embryos of the tobacco budworm, *H. virescens*. The morphologies of these cells are seen in Fig. 1. HVE1a, HVE1-It, and HVE6a are each predominantly fibroblast like, firmly attached cells although the HVE1a and HVE1-It cells are narrow and form a web-like network (Figs. 1A and 1B) while the HVE6a cells are broader and are closely aligned to each other in parallel arrays (Fig. 1C). Twenty to 30% of HVE6a-It are similar to HVE6a but at least 70% of these cells exhibit epithelial-like morphology (the ratio of the two cell types varies somewhat with the age of the culture) (Fig. 1D). HVE1s cells are predominantly round and suspended and occur singly or in small clumps (Fig. 1E), while both HVE6s and HVE6s-It are large aggregates of suspended cells with some cells occurring as well organized vesicles (Fig. 1F). The low temperature strains were selected because cell lines may develop with a broader host range to various insect viruses at low temperature (Winstanley and Crook, 1988).

The similarity of the isozyme patterns of these cells to HvT1, another *H. virescens* line maintained in our lab, indicates that they are derived from *H. virescens* (Fig. 2). Of the four systems used, the migration of ME and PGI enzymes was identical among all seven new strains and HvT1. One of two allozymes present in the PGM gels differed between the new strains and HvT1, possibly due to differences in the source of the originating insects or the different tissue source of the cells. In addition to the migration of this allozyme differing slightly between the new strains and HvT1, it was also much less intense with the three HVE1 strains. Interestingly, no ICD enzyme activity was detected in any of the low-temperature strains. The PGM isozyme patterns of the new strains were similar to patterns obtained with several *Trichoplusia ni* cell lines (data not shown) but

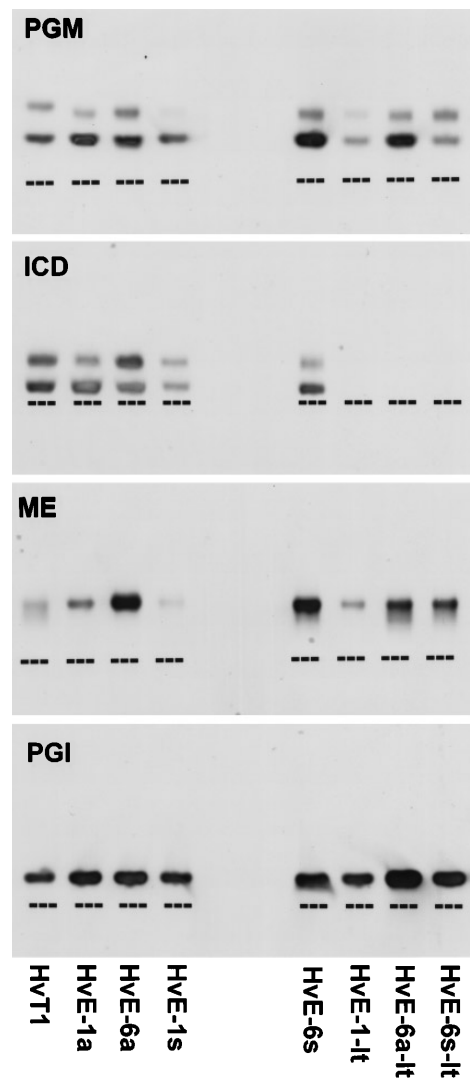


FIG. 2. Electrophoresis patterns on gels stained for phosphoglucotomutase (PGM), isocitrate dehydrogenase (ICD), malic enzyme (ME), and phosphoglucose isomerase (PGI). All materials were from the Authentikit system. The triple dash mark (---) shows the location where the individual cell extracts (from the cell line indicated at the bottom of the figure) were applied to the gels. Samples were electrophoresed 25 min prior to staining with the indicated substrate.

none of the other enzymes matched these or any other lines maintained in our laboratory. Based on the isozyme results, we are confident that the strains described in this paper are unique.

All of the new strains were highly susceptible to the five noctuid viruses tested (AcMNPV, AfMNPV, AgMNPV, HzSNPV, and RoMNPV) while being only slightly susceptible to OpSNPV and showing no cytopathology with OIMNPV or LdMNPV (Table 1). Of these, the replication of AgMNPV was most surprising. With the exception of cells from the same species (UFL-AG286, Sieburth and Maruniak, 1988), it replicates quite poorly in most of the cell lines maintained in our

TABLE 1

Replication of Various Nucleopolyhedroviruses in New Cell Strains from *H. virescens*

Virus	Cell lines						
	HvE1a	HvE1s	HvE1- lt	HvE6a	HvE6a- lt	HvE6s	HvE6s- lt
HzSNPV	+++	(+)	+++	+++	+++	+++	+++
AcMNPV	++	++	++	+++	++	+++	++
AfMNPV	+++	+++	++	+++	+++	++	++
RoMNPV	++	+++	++	+++	+++	++	++
LdMNPV	-	nt	-	-	-	-	-
OIMNPV	-	-	-	-	-	-	-
OpMNPV	-	nt	+	+	+	-	-
AgMNPV	+++	+++	+++	+++	+++	+++	+++

Note. +++, over 90% of cells with CPE (OBs); ++, 50–80%; +, 10–20%. nt, not tested. -, no CPE apparent.

laboratory but produces as many OBs in at least two of the new strains as UFL-AG286 (unpublished preliminary data).

The quantitative studies with HzSNPV revealed that several of the new strains were capable of producing more OBs than the previously available HvT1 line (Fig. 3). As mentioned earlier, the clumping of several of these strains makes counting these cells difficult. For this reason, the production of OBs is presented on a per milliliter basis. In assaying the various strains for HzSNPV production, three cell densities (representing 0.5, 1, and 2× the normal number used to initiate cultures) were tested with a constant amount of virus. In these experiments, the two attached, 26°C strains, HvE1a and HvE6a, produced the most OBs. As with other cell lines that we have compared (Lynn *et al.*,

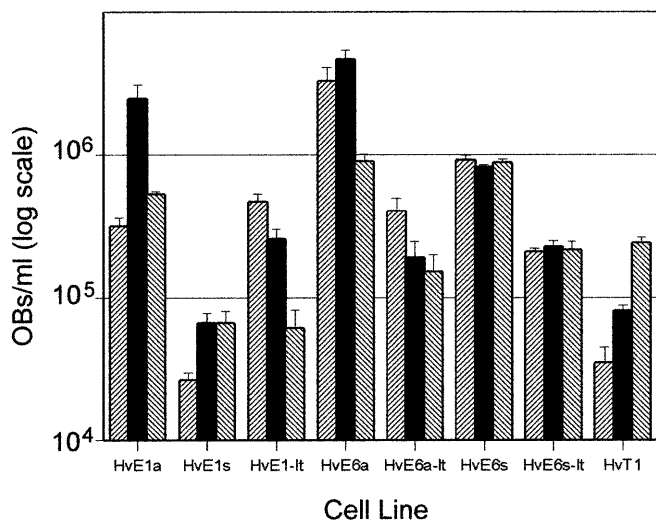


FIG. 3. Production of HzSNPV occlusion bodies (OBs) in new cell strains. The first bar in each series is from cells that were set up at half the normal density, the middle bar (solid black) was set up at the normal density, and the third bar was set up at twice the normal density. The number of OBs was counted by lysing cells 9 days postinfection. Each bar is the mean of three replicate experiments.

1989; Lynn and Shapiro, 1997), there is no good explanation as to why different cell lines produce variable amounts of virus. We suspect that this ultimately relates to the actual cell type represented by the cultured cells since it is clear that different tissues in the insect become infected at different times and produce different quantities of virus. We are currently developing an assay for the nonoccluded virus so we can determine if there are also quantitative differences with that form of the virus. These new strains appear to have good potential for studying HzMNPV and, with some further optimization of the growth conditions, they may also be useful in a production scheme for this virus. We are also currently examining the potential of these cells for replicating a granulosis from *Helicoverpa armigera*.

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