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Propagation of *Bombyx mori* Nucleopolyhedrovirus in Nonpermissive Insect Cell Lines

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This study addresses the susceptibility of *Spodoptera frugiperda* (Sf9 and Sf21), *Trichoplusia ni* (Hi5), and *S. exigua* (Se301) cells to the *Bombyx mori* nucleopolyhedrovirus (BmNPV). Although these cells have classically been considered nonpermissive to BmNPV, the cytopathic effect, an increase in viral yield, and viral DNA synthesis by BmNPV were observed in Sf9, Sf21, and Hi5 cells, but not in Se301 cells. Very late gene expression by BmNPV in these cell lines was also detected via β-galactosidase expression under the control of the polyhedrin promoter. Sf9 cells were most susceptible to BmNPV in all respects, followed by Sf21 and Hi5 cells in decreasing order, while the Se301 cells evidenced no distinct viral replication. This particular difference in viral susceptibility in each of the cell lines can be utilized for our understanding of the mechanisms underlying the host specificity of NPVs.

Keywords: *Bombyx mori* nucleopolyhedrovirus, susceptibility, nonpermissive insect cell, host specificity

The Baculoviridae are characterized by enveloped rod-like nucleocapsids, and include both the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs). Baculoviral infections have been detected in more than 600 insect species in the orders Hymenoptera, Diptera, Coleoptera, Neuroptera, Trichoptera, and Thysanura, as well as in the Crustacean order Decapoda (Murphy *et al.,* 1995). The NPVs have been investigated extensively, because of their potential as an insect pest control agent and as a vector for the expression of a variety of heterologous genes. The large number of review papers and books thus far published on the subject are reflective of the broad general interest in baculoviruses (Adams and McClintock, 1991; O'Reilly *et al.,* 1992; Inceoglu *et al.,* 2001; Kost *et al.,* 2005).

NPVs are generally quite host-specific, but little has been determined thus far regarding the mechanisms underlying the host specificity of NPVs. Infection by the majority of NPVs is limited to a single species or to a few closely related insect species. This characteristic has limited the usefulness of the NPVs as a pesticide. In order to circumvent this problem, a variety of studies have been conducted regarding the initial attachment of the virus, viral penetration into the nucleus, DNA replication, the construction of a host range-expanded recombinant virus, etc (Lenz, 1990; Kondo and Maeda, 1991; Croizier *et al.*, 1994; Thiem *et al.*, 1996). Several studies regarding viral replication have demonstrated that these viruses are able to enter and express some genes, although they do not replicate in insect cells, which are nonpermissive (Morris and Miller, 1992). A few baculovirus genes that affect the host range of the virus have been also identified. P35 and apoptosis inhibitor prevent the programmed cell death of baculovirus-infected cells (Clem *et al.,* 1991; Crook *et al.,* 1993; Kamita *et al.,* 1993). P143, a baculovirus-encoded protein that is homologous to the DNA helicase, also affects viral host range (Lu and Carstens, 1991). A recombinant virus, between a small region of the DNA helicase genes of *Bombyx mori* NPV (BmNPV) and *Autographa californica* NPV (AcMNPV), evidenced an expanded host range (Kondo and Maeda, 1991; Croizier *et al.,* 1994), but the exact mechanism by which this occurs remains unknown. Comparisons of AcMNPV and BmNPV replication in cells exhibiting different degrees of permissivity constitute an attractive model for investigations of the genetic determinants of host specificity (Croizier *et al.,* 1994). *Spodoptera frugiperda* cells have been classically considered to be nonpermissive to BmNPV and permissive to AcMNPV, whereas *B. mori* cells are considered to be nonpermissive to AcMNPV and permissive to BmNPV (Kondo and Maeda, 1991; Croizier *et al.,* 1994). Despite their unique host specificities, the genomes of BmNPV and AcMNPV evidence relatively high levels of DNA homology, as shown by the results of DNA hybridization and direct sequencing experiments (Ayres *et al.,* 1994; Gomi *et al.,* 1999). A suite of 18 late expression factors (lefs) is required for the expression of late AcMNPV genes in Sf21 cells (Lu and Miller, 1995; Todd *et al.,* 1995). P35 and p143 are among these lefs. Different lefs may be required for viral replication in different cell types, thus influencing virus host ranges. Recently, a study was conducted concerning the infection of *S. frugiperda* cells by BmNPV (Huang *et al.,*

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1997; Martin and Croizier, 1997). This report indicated DNA replication and an increasing infectious titer of BmNPV in Sf9 cells. However, they were unable to observe any discernible shutoff of cellular protein synthesis, overt cytopathic effects, or polyhedral formation via the expression of the very late gene.

In this study, we have evaluated the characteristics of BmNPV replication using a recombinant virus harboring the lacZ gene in its different nonpermissive cell lines, Sf9, Sf21, Se301, and BTI-Tn-5B1-4 (Hi5), and have demonstrated its replication and the expression of the very late gene.

Materials and Methods

Cell cultures and viruses

S. frugiperda Sf9 and Sf21, *B. mori* Bm5 and *Trichoplusia ni* BTI-Tn-5B-4 (Hi5) cells were maintained at 27°C in TC-100 medium supplemented with 10% fetal bovine serum (FBS).

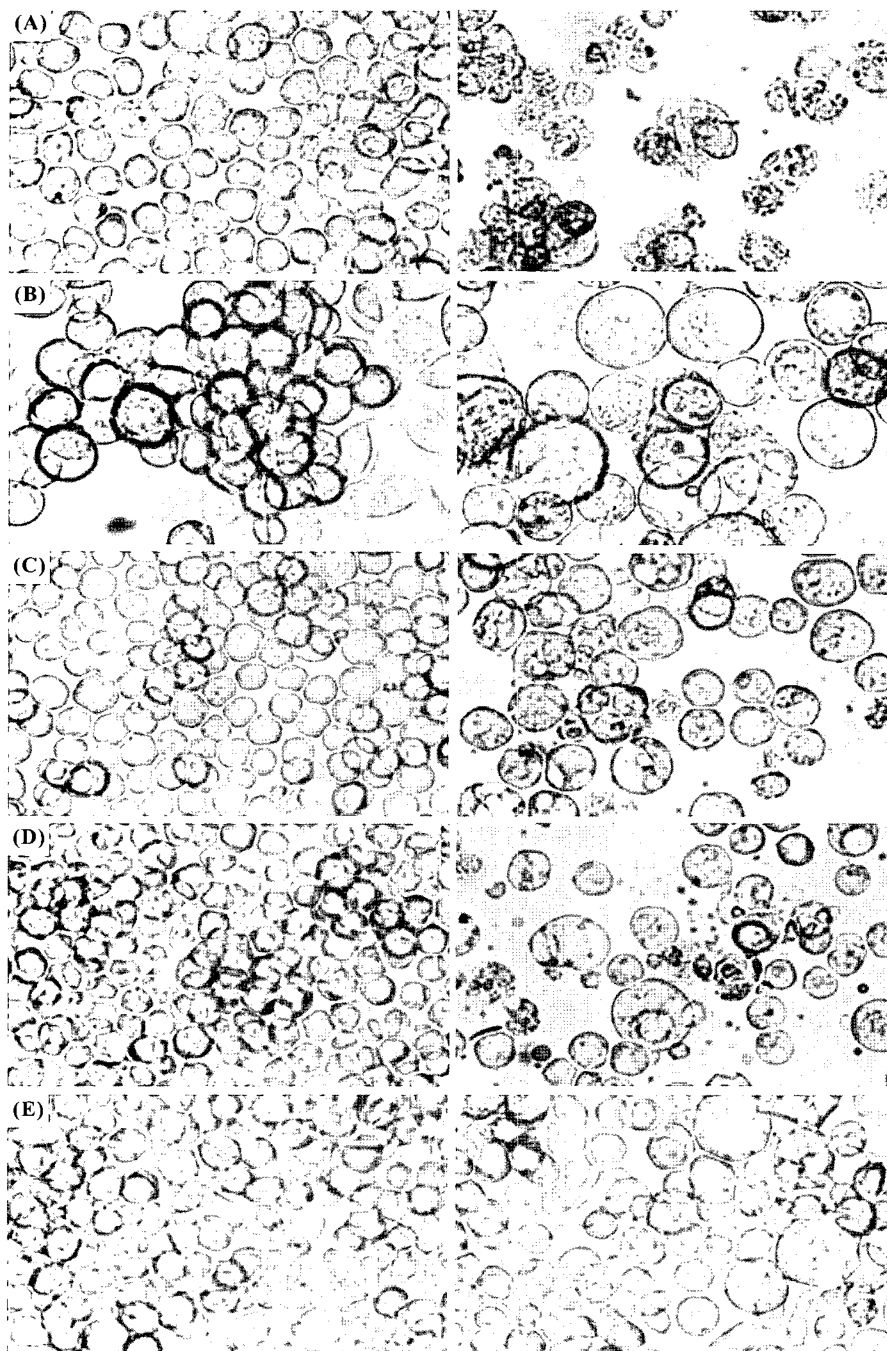

Fig. 1. Morphology of various cell lines, Bm5 (A), Hi5 (B), Sf9 (C), Sf21 (D), and Se301 (E) cells, at 5 days after mock-infection (left panel) or infection with BmK1-LacZ at a MOI of 10 PFU/cell (right panel). 200X.

S. exigua Se301 cells were maintained in IPL-41 medium supplemented with 10% FBS. BmK1-lacZ, a recombinant BmNPV strain (BmNPV-K1) (Hong *et al.*, 2000) harboring the *E. coli* lacZ gene rather than the polyhedrin gene, was propagated in Bm5 cells. Routine cell culture maintenance and virus production protocols were followed, in accordance with previously published procedures (O'Reilly *et al.*, 1992).

Virus infection and β-galactosidase assay

A variety of cultured cells were infected with BmK1-lacZ at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell in 6-well culture plates, each containing 1.0 $\times 10^6$ cells/well. After a 1 h viral adsorption period, the cells were washed, supplied with fresh medium, and incubated at a temperature of 27°C. At various times (0 to 7 days) following inoculation, the culture supernatants were harvested and pelleted. The cell pellets were disrupted via repeated freeze-thawing. After the cellular debris had been pelleted, the extracts were assayed for β-galactosidase activity using the o-nitrophenyl-β-D-galactopyranoside with the β-Galactosidase Enzyme Assay System (Promega), and a model 550 microplate reader (Bio-Rad).

Viral yield and dot blot analysis of the viral DNA

In order to estimate the viral yield, aliquots were sampled at appropriate times after viral infection and the viral titers were determined via the end-point dilution method (O'Reilly *et al.*, 1992) in Bm5 cells. For dot blot analysis, a total of 10^6 cells infected with BmK1-lacZ were lysed at the indicated time points, and the DNA was dot-blotted onto a nitrocellulose membrane and probed with random priming pBmKSK1-LacZ, the transfer vector of BmNPV harboring the lacZ gene.

SDS-PAGE and Western blot hybridization

The total protein content of the virally-infected cells was then determined via SDS-PAGE. After electrophoresis, the separated proteins were transferred electrophoretically to a nylon membrane and immunoblotted with mouse anti-β-galactosidase polyclonal antibody. The membrane was then incubated with diluted goat anti-mouse IgG conjugated to alkaline phosphatase. Bound antibodies were detected via the incubation of the membrane with substrate solution (5-bromo-4-chloroindoxyl phosphate and nitroblue tetrazolium).

Results

Cell morphology

In order to observe and assess any cytopathic effects, we assessed the morphology of the virally-infected cells for 7 days. The results at 5 days post infection (p.i.) are shown in Fig. 1. Infection of the permissive Bm5 cells with BmK1-lacZ resulted in a pronounced cytopathic effect by 2 days p.i. At 5 days p.i., the majority of the Bm5 cells had become detached from the culture plates and disrupted (Fig. 1A). When nonpermissive cell lines, including Hi5, Sf9, and Sf21, were infected, cytopathic effects were also detected in all of them. The infected Hi5 cells were round in shape, and developed nuclear hypertrophy by 2 days p.i. By 5 days p.i., the majority of the Hi5 cells were detached from the plate and not aggregated, unlike what was observed in the mock-infected cells (Fig. 1B). Although cell lysis via viral infection was observed by 6 days p.i., it was

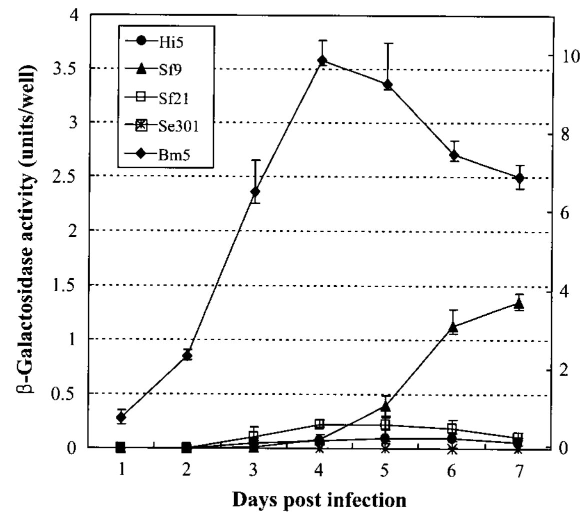

Fig. 2. β-Galactosidase activity assays in each BmK1-LacZ-infected cell line. At various times after inoculation, the culture supernatant was harvested and pelleted. The cell pellet was washed with phosphate-buffered saline (pH 6.2), and the cell extracts were assayed for β-galactosidase activity with o-nitrophenyl-β-D-galactopyranoside using a β-Galactosidase Enzyme Assay System. The right scale is applicable only to Bm5.

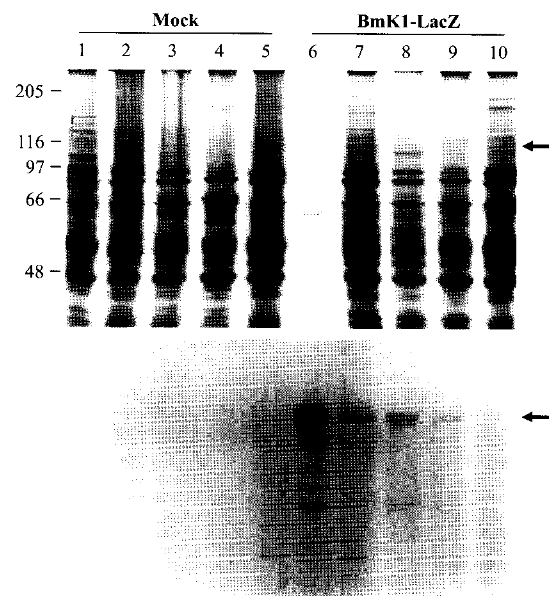

Fig. 3. SDS-PAGE (upper panel) and Western blot analysis (lower panel) of β-galactosidase in a variety of cell lines after mock-infection or BmK1-LacZ infection. Proteins from Bm5 (lanes 1 and 6), Hi5 (lanes 2 and 7), Sf9 (lanes 3 and 8), Sf21 (lanes 4 and 9), and Se301 (lanes 5 and 10) cells were isolated at 5 days post-infection, electrophoresed on 10% SDS-polyacrylamide gel, blotted onto nylon membranes, and allowed to react with anti-β-galactosidase polyclonal antibody. The position of the protein molecular mass standards is shown on the left in kilodaltons. Arrows indicate the β-galactosidase protein band.

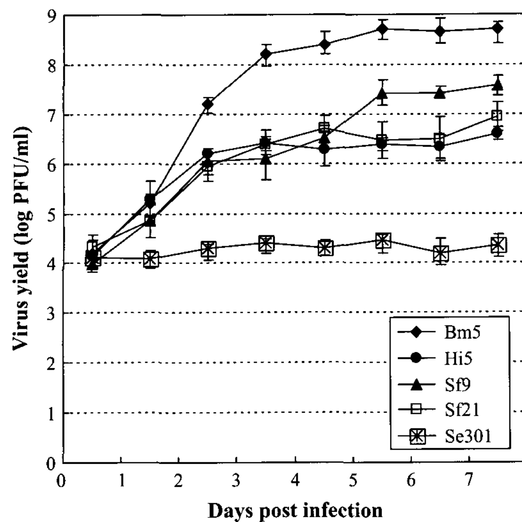

Fig. 4. Infectious viral yields from BmK1-LacZ-infected nonpermissive cells. Aliquots were sampled at indicated times after infection, and the viral titers were determined via end-point dilution on Bm5 cells.

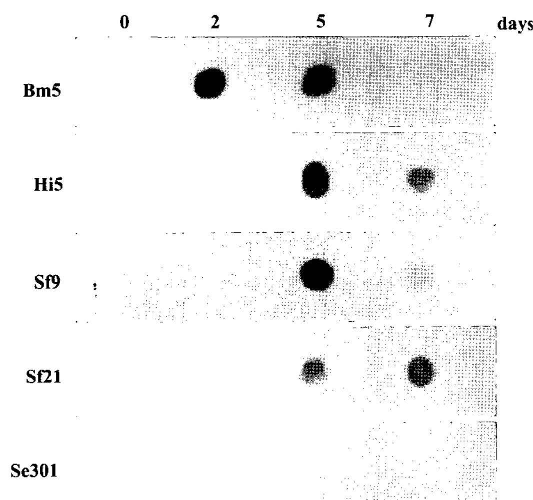

Fig. 5. Viral DNA synthesis in BmK1-lacZ infections of Bm5, Hi5, Sf9, Sf21, and Se301 cells at 0, 2, 5, and 7 days post-infection. DNA from each cell line was isolated, dot blotted, and hybridized using labeled pBmKSK1-lacZ probes.

limited to a small number of cells until 7 days p.i. The infected Sf9 cells were also beginning to detach from the matrix, and took on a granular appearance by 5 days p.i. (Fig. 1C). The majority of the Sf9 cells were disrupted by 7 days p.i.. By way of contrast, the infected Sf21 cells evidenced earlier nuclear hypertrophy, became detached from the matrix, and disintegrated significantly earlier than other nonpermissive cell lines (Fig. 1D). In addition, the numbers of Sf21 cells did not increase to a substantial degree after 2 days p.i.. The infected Se301 cells did not differ significantly from the mock-infected cells. Only a few cells evidenced hypertrophy by 5 days p.i. (Fig. 1E).

β-Galactosidase production

In order to assess the β-galactosidase activity generated by BmK1-lacZ, samples from a variety of infected cells were collected at 1-day intervals. Fig. 2 shows the activity of β-galactosidase expression in the infected cells. Although the β-galactosidase activity was quite low when comparing the Bm5 cells, the Sf9 cells evidenced the highest levels, followed by Sf21 and Hi5 cells (in decreasing order) among the nonpermissive cells, whereas the Se301 cells evidenced no detectable activity. Protein accumulation in the infected cells at 5 days p.i. was compared via SDS-PAGE and Western blot analyses (Fig. 3). The protein synthesis profiles of nonpermissive cells infected with BmK1-lacZ evidenced no significant decline, as compared with the Bm5-infected cells. We were unable to distinguish any expressed β-galactosidase protein bands in the nonpermissive cells on SDS-PAGE. However, Western blot analysis using anti-β-galactosidase polyclonal antibody evidenced a band of approximately 116 kDa corresponding to the β-galactosidase protein, which was indicative of β-galactosidase expression in the Sf9, Sf21, and Hi5 cells. The Se301 cells evidenced no detectable β-galactosidase protein synthesis.

Virus growth

Viral growth patterns were evaluated using the Bm5 cell line. As is shown in Fig. 4, the titer of budded virus (BV) increased by about 30,000-, 2,000-, 400-, and 200-fold during infection of Bm5, Sf9, Sf21, and Hi5 cells, respectively, by 7 days p.i., whereas a similar infection of Se301 cells resulted in no significant increases. The low BV titer observed in the Se301 cells compelled us to analyze the relative levels of viral DNA synthesized in each of the cell lines. The results of this showed that viral DNA was undetectable in all cell lines at 0 days p.i. However, detectable viral DNA synthesis levels were present in the Hi5, Sf9, and Sf21 cells at 5 days p.i. (Fig. 5). We detected no viral DNA synthesis in the Se301 cells.

Discussion

The susceptibility of four lepidopteran cell lines to BmNPV were evaluated in terms of cytopathic effects, infectious virus yield, viral DNA replication, and foreign gene expression by the very late gene promoter. Three nonpermissive cell lines, Sf9, Sf21, and Hi5, evidenced typical cytopathic effects, including nuclear hypertrophy and matrix detachment. These observations were corroborated by the infectious virus yields and the results of DNA replication analyses from the respective cell lines. Basal viral titers were approximately 10^4 PFU/ml in all cell lines. Following infection, the titers in Sf9, Sf21, and Hi5 reached levels of 10^6 or 10^7 PFU/ml, and an increase in DNA synthesis was also observed. Although these characteristics were observed later than in the Bm5 cells, the results indicate that BmNPV can replicate in Sf9, Sf21, and Hi5 cell lines, and that Sf9 is the most susceptible by all measured aspects. The infectivity of BmNPV, which is generated in nonpermissive cell lines, was confirmed in the Bm5 cells (data not shown). In the first report regarding BmNPV replication in a nonpermissive cell line, Martin and Croizier (1997) demonstrated BmNPV

DNA replication and increasing viral titer in the Sf9 cells. However, they observed no overt cytopathic effects and low infectious viral yield levels. By way of contrast, we observed pronounced cytopathic effects, an increase in infectious virus yield, and DNA synthesis occurring in the Sf21 and Hi5 cells, including the Sf9 cells.

This report, to the best of our knowledge, is the first to address BmNPV replication in Sf21 and Hi5 cells. The absence of viral DNA synthesis in Se301 cells as observed herein is consistent with recent observations that the host-specific restriction of baculovirus replication is principally attributable to the inability of viral DNA to replicate efficiently, although restrictions at other stages can not be ruled out (Lenz, 1990; Thiem *et al.*, 1996). The exact mechanism of host specificity at the DNA replication level remains unclear. Reduced viral DNA synthesis in the Bm5 and Hi5 cells at 7 days p.i. (Fig. 5) is believed to be associated with the lysis of virally-infected cells. We assessed the initial viral infection efficiency of each cell line in order to determine the reasons for these observed differences in viral yields. The wash from media immediately after the induction of viral infection was employed to determine the initial viral infection rate. The initial viral infection rates, which was calculated retroactively from the ratio of collected inoculum viral yields to the initial inoculum viral yields in the Hi5, Sf9 and Sf21 cells, were 54, 57, and 33%, respectively (data not shown). We detected no correlation between initial virus infection efficiency and overall viral yield. However, the Se301 cells evidenced an infection rate of 16%, thus indicating that this may influence the degree of virus replication. Although the same cell line, Sf9, was utilized in previous studies (Martin and Croizier, 1997) as well as in this one, an overt cytopathic effect was observed only in this study. This may be attributable to differences in the viral strains utilized (Hong *et al.*, 2000). However, we consider the difference in infectious virus yield between the two studies (Martin and Croizier, 1997), to be attributable not only to the viral strains, but also to the method by which viral yield titers were determined. While in previous studies, viral titer and other viral characteristics were evaluated on the basis of occlusion body formation (Kondo and Maeda, 1991; Martin and Croizier, 1997), we assessed these characteristics in terms of the expression of the lacZ gene, which is more sensitive than the detection of occlusion body formation. This may result in the higher viral yields observed in our experiments. In fact, we were able to observe occlusion body formation in Sf9 cells infected with wild-type BmNPV K1 at 7 days p.i., but this was limited only to the production of a few polyhedra (data not shown). This was also observed when examining very late gene expression. We observed β-galactosidase expression by a polyhedrin promoter in nonpermissive cells, but in the previous report (Martin and Croizier, 1997), we observed no expression of the very late gene, including the production of polyhedra. This difference may be attributed to the profound difficulty inherent to the observation of polyhedra formation due to low polyhedrin expression, as well as to differences between viral species. Although Sf9, Sf21, Hi5, and Se301 cells are highly susceptible to AcMNPV, interestingly, only the Se301 cells proved not to be susceptible to BmNPV.

Studies of baculovirus host specificity have previously demonstrated that the majority of NPVs evidence a relatively narrow host range, both *in vivo* and *in vitro*. In general, NPVs infect only members of the genus or, in some cases, the family of the original host. AcMNPV, however, exhibits a broad host range and can successfully infect more than 25 species of insects. It can replicate in many cell lines derived from six families of Lepidoptera. *S. frugiperda*, *T. ni*, and *S. exigua* are members of the Noctuidae superfamily, and are consequently susceptible to AcMNPV (Adams and McClintock, 1991). However, as *B. mori* is in the Bombycoidea superfamily, its viral susceptibility also differs from that of others. In this study, the tested nonpermissive cell lines were susceptible to BmNPV, with the exception of the Se301 cells, which indicates that AcMNPV and BmNPV share the factor(s) of host specificity, but that more factor(s) may be required, depending on the cell line. This is corroborated by comparisons of previous reports (Kondo and Maeda, 1991; Mukherjee *et al.*, 1995) with our results; the level of AcMNPV replication in *B. mori* cell lines is similar to that of BmNPV replication in AcMNPV-permissive cell lines. Continuing studies targeted toward a determination of the idiosyncratic differences in each cell line may prove useful with regard to our understanding of the host specificity of NPVs.

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