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Productive replication of *Malacosoma neustria* nucleopolyhedrovirus (ManeNPV) in Md203 cell line

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Abstract: A plaque-purified genotypic variant, derived from a field isolate of the *Malacosoma neustria* nucleopolyhedrovirus (ManeNPV) from Turkey was characterized based on in vitro replication properties in a cell line, Md203 derived from *Malacosoma disstria*. The life cycle of ManeNPV was studied based on the cytopathic effects (CPEs), polyhedral inclusion body (PIB) formation, budded virus (BV) production, viral DNA replication and polyhedrin protein expression in ManeNPV-infected Md203 cells. Infection of Md203 cells with ManeNPV resulted in cytopathic effects within 24 and 36 h post infection and most cells detachted from the bottom of the culture plate on the fourth day. Typical NPV cytopathic effects (CPE) like granulated and rounded cells, nuclear hypertrophy and impairment in cell proliferation, and lost cell shape with the extendings were observed. Budded viruses were detected at 24 h p.i. However, significant increase in BV production was observed after 48 h p.i. PIB was firstly detected at 36 h p.i. When BV's were used for reinfection of the Md203 cells, the result showed that these cells support production of viable virus progeny. We also observed a significant level of viral DNA synthesis by 24 h p.i. The results indicate that ManeNPV can be propagated at in vitro system for further studies and thus has a potential for development into an effective biotechnological and microbial insecticidal agent.

Key words: Baculovirus, *Malacosoma neustria* NPV, virus replication, Md203 cell line

Malacosoma neustria nukleopolihedrovirus (ManeNPV)'unun Md203 hücre şusunda üretken replikasyonu

Özet: Türkiye'de araziden toplanan *Malacosoma neustria* larvalarından izole edilen *Malacosoma neustria* nukleopolihedrovirus (ManeNPV) plak yöntemiyle saflaştırıldı. Elde edilen genetik varyantın in vitro replikasyonal özellikleri *Malacosoma disstria*'dan geliştirilmiş Md203 hücre suşunda karakterize edildi. ManeNPV'nin Md203 hücrelerindeki replikasyon döngüsü sitopatik etkilerin (CPEs) tespiti, polihedral inklüzyon yapıların (PIB) oluşumu, tomurcuk virusların (BV) üretimi, viral DNA'nın replikasyonu ve polihedrin proteinin ifadesine göre çalışıldı. Enfeksiyondan sonra 24 ve 36 saat içinde ManeNPV ile enfekte Md203 hücrelerinde sitopatik etkilerin oluştuğu ve enfeksiyonun 4. gününde hücrelerin çoğunun kültür kabı tabanından ayrıldığı tespit edildi. Enfekte hücrelerde granülleşme, sitoplazmik uzantılarını kaybederek yuvarlaklaşma, nukleus irileşmesi ve bölünen hücre sayısının azalması gibi tipik NPV sitopatik etkileri gözlendi. Ektrasellular virus üretimi enfeksiyondan 24 saat sonra başladı. Ancak, BV miktarındaki önemli artışın enfeksiyondan 48 saat sonra meydana geldiği belirlendi. İlk PIB oluşumu ise enfeksiyondan 36 saat sonra tespit edildi. Md203 hücreleri BV'ler ile yeniden enfekte edildiklerinde hücrelerde yavru virus oluştuğu belirlendi. Ayrıca, enfeksiyondan 24 saat sonra viral DNA sentezinin önemli seviyede arttığı gözlendi. Sonuçlar, ManeNPV'nin ilerki çalışmalar için in vitro sistemde çoğaltılabileceğini ve böylece, virusun etkili bir biyoteknolojik materyal ve mikrobiyal insektisid haline dönüştürülebilme potansiyeline sahip olduğunu göstermektedir.

Anahtar sözcükler: Bakulovirus, *Malacosoma neustria* NPV, virus replikasyonu, Md203 hücre suşu

Introduction

Nucleopolyhedroviruses (NPVs) belonging to the family *Baculoviridae*, are one of the most promising biological and biotechnology material to date. Baculoviruses are enveloped viruses that have double-stranded, circular DNA genomes ranging in size from 80-180 kbp (1,2). These viruses has been used as biopesticides to control the population of insect pest of agriculture, forestry, and pasture (3-5). Recombinant baculoviruses are being used at biotechnology for the expression of interested genes proteins under the control of strong *polh* and *p10* genes promotors (6-8). At medical area, they attracted attention as gene therapy vectors (9,10). Also baculoviruses are model organisms at molecular biology studies in gene structure.

Baculoviruses play an important ecological role regulating the size of insect populations (11). These viruses occur naturally in insect populations and are capable of causing very high mortality. NPVs have been isolated from more than 800 species that span *Lepidoptera*, *Hymenoptera*, *Diptera*, *Orthoptera*, *Coleoptera*, *Neuroptera*, *Thysanera*, and *Trichoptera* orders (4,12,13). Previous studies which examined cross-infectivity in insect larvae have collectively indicated that each of these NPV isolates exhibited a unique host range which was confined in most cases, to a few, if not a single, insect species belonging to the same family. 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 (14). In addition, insect cell lines have been used for studying viruses almost since the first continuous lines were established. Up to now, many baculoviruses have been replicated in a wide variety of lepidpoteran cell lines (15,16). Studies with cultured cells have demonstrated that NPVs establish unique interactions with different cell lines, resulting in various types of productive and non-productive infection (15-17).

The family *Baculoviridae* is divided into two genera based on occlusion body (OB): the nucleopolyhedroviruses (NPVs) and granuloviruses (GVs) (18). Baculoviruses have a unique replication. There are two infectious forms in the baculoviruses. One is known as budded virus (BV) and provide the transmission of infection from cell to cell both in host systematically and cultured insect cell lines. The other one occlude in a proteinaceous crystalline and provide the circulation of infection from larvae to larvae in the environmet and also known as polyhedral occlusion bodies (OD).

Up to now, a few nucleopolyhedrovirus were isolated from *Malacosoma neustria*, and their biological and morphological characteristics, and ecological and molecular properties have been determined (19-21). However, nothing is known about the replication cycle and biological properties of this virus in cell culture system. Mitsuhashi (22) showed that *M. neustria testacea* NPV infected a continuous cell line from larval hemocytes of *M. neustria testacea*, but, he did not declare any other information about the details of this infection. It is important to study detailed replication properties of ManeNPV to make it a valuable tool for microbiol control and biotecnology.

In this study, *M. neustria* nucleopolyhedrovirus (ManeNPV) which was isolated from the larvae of *Malacosoma neustria* in north-east of Turkey was studied in cell culture system. The replication strategy of the virus was examined. In order to develop useful host specificity models and to determine the host range of novel ManeNPV, budded virus was isolated from larval hemocytes of infected *M. neustria* larvae. Md203 cell line was infected with this virus. Formation of CPEs, budded virus and polyhedral inclusion body, DNA replication and protein synthesis of ManeNPV at this infection were investigated.

Materials and methods

Cell line and virus

In this study UA-Md203 cell line established from larval hemocytes of *Malacosoma disstria* by Keddie et al. (23) was used. Cells were grown at 27 °C in TNMFH (Sigma) medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma) and 0.25 g/100 ml tryptose broth (Sigma) and in 25 cm^2 tissue culture flasks (Grainer) at 5-day intervals at 1:5 ratio.

ManeNPV was isolated from *Malacosoma neustria* larvae collected from Gumushane, Turkey, in May in 2006.

Insect

Malacosoma neustria larvae were collected from natural plants from agricultural fields. They were reared on appropriate foliage in the laboratory at 25 °C until they were used in the study.

Preparation of primer viral inoculum from infected larvae

ManeNPV inoculum for Md203 cell line was prepared from infected *M. neustria* larvae according to Mitsuhashi (24) and Lavina et al. (25) with some modifications. Fourth instar *M. neustri* larvae were infected with OBs collected from cadavers which died because of ManeNPV. Infection was performed by feeding the larvae with virus contaminated leaves. After 4 days post infection (p.i.), the infected larvae were surface-sterilized by dipping briefly into 70% ethanol and allowed to dry on steril filter paper. A proleg was amputated with a surgical blade, and the hemolymph was collected into microcentrifuge tube containing aliquots phenyl thiocarbamide on ice to prevent melanization. The hemolymph was diluted with an equal volume of TNMFH medium and then centrifuged at 2,000 X g for 10 min. at 4 °C to separate cells from hemolymph. Pellet was resuspended in 500 µl medium and filter-sterilized through a 0.45µm membran filter attached to a syringe. The filtrate was stored at -80 °C.

Infection of cells by extracellular virus and plaque purification

The monolayer of Md203 cells in 25 cm^2 culture flask was infected with the filtrate from the infected *M. neustria* larvae and incubated at 27 °C. When it is detected that 80% of the cells contain PIB, the culture media including extracullar virus was collected and centrifuged at 2,000 X g for 10 min. to remove cells and viral occlusion body (OB). The supernatant was used to provide plaque purified ManeNPV.

Serial dilutions prepared from supernatant were used for the infection of Md203 cell line. Cells were seeded at 1 X 10^6 concentration to 35-mm petri dishes and allowed for attaching to dishes for 1 h. The cells were inoculated with 200 µl of virus supernatant. After 1 h adsorption, viral inoculum was removed and cells were overlaid with 3 ml (per dish) of 0.75% SeaPlaque agarose. The dishes were sealed with parafilm and incubated at 27 °C for 5 days or until plaques became clearly visible. The plaques were picked with Pasteur pipettes and suspanded in 500 µl TNMFH medium. Plaque purified ManeNPV was used for reinfection of the cells and accepted as passage one to produce stock virus. The concentration of the stock virus was determined by end point dilution titration ($TCID_{50}$) (26).

Infection of cells with ManeNPV

Md203 cells were seeded at a density of 1 X 10^6 cell/well to 6-well culture plates and infected with ManeNPV at an MOI of 10 PFU/ml. After incubation for 2 h the infectious medium was removed and cells were washed twice with their respective medium, and overlaid with 2.5 ml of fresh medium. The time when the fresh medium was added prior to incubation was designated as 0 h p.i. Mock-infected cells were prepared in the same manner using the medium as the inoculum. Cells were incubated at 27 °C.

One batch of infection was examined to determine cytophatic effects (CPEs) under phase-contrast microscopy (Nikon), and photographed at 12 h intervals until 96 h p.i. The other batch of infection was used for the analysis of budded virus production. For this purpose the medium from infected cells was harvested at 24 h intervals until 96 h p.i. by centrifugation at 2,000 X g for 10 min, and supernatants were stored at 4 °C. The titer of extracellular virus in supernatants was determined by $TCID_{50}$ (26).

Slot blot analysis of viral DNA replication

Slot blot analysis was performed to determine if ManeNPV DNA entered into and/or replicated in the *M. disstria* (Md203) cell line. That was realized on a few steps explained below.

DNA isolation of ManeNPV for prob preparation

Cells were infected with ManeNPV at MOI 10 as above. After 3 day p.i, viral DNA was isolated from ManeNPV-infected cells according to the protocol by Invitrogen (27). Briefly, cells were centrifuged at 5,000 rpm for 3 min, supernatant was transfered to 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 ethanol precipitaion was performed. Finally, the pellet was washed with 70% ethanol, resuspended in 10 ml ddH_2O, and stored at 4 °C.

Prob preparation for polh gene

A *polh* fragment of ManeNPV DNA was amplified by using specific primers for *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') from this DNA in a total volume of 50 ml by polymerase chain reaction (PCR) (28). Reaction mixture included 4 µl of ManeNPV DNA, 5 µl (10 X) PCR buffer, l 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 (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 following conditions; l 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 cleaned from gel. DNA fragments were then labeled with DIG according to the manufacturer's (Roche) instructions and labeled probe was stored at -20 °C until used.

Preparation of samples and Slot blot hybridization

Md203 cells were seeded at a density of l X $l0^6$ cell/well to a 6-well plate (Grainer), and infected with ManeNPV at MOI of 10. Culture medium was removed at 0, 2, 4, 6, 8, 10, 12, 24, 48 and 72 h p.i. and cells were lysed by adding 800 ml of 0.5 N NaOH into each well. The suspension was neutralized with 80 ml of 10 M NH_4-acetate. 100 ml of each cell lysate's were applied to the immobilon transfer membrane (Millipore) under vacuum. The hybridization of the membrane with labeled *polh* probe was carried out by using the Dig High Prime DNA Labeling and Detection Starter Kit I (Roche).

Detection of virus specific protein (ICSP) synthesis in infected cells

Md203 cells in a 6-well plate (l X $l0^6$ cells/well) were infected with ManeNPV at MOI of 10. The infected and mock-infected cells were harvested at 12 h intervals until 96 h p.i. by centrifugation at 5,000 rpm for 10 min at 4 °C and lysed using lysis buffer (0.1% Triton X-100 including 50 µg/ml leupeptin and 100 µg/ml pepsitatin A, prepared in PBS). Protein extraction procedures were followed as described by O'Reilly et al. (6). Proteins were electrophoresed by 10% SDS-PAGE at 30 mA according to Laemmli (29), and stained with Coomassie brillant blue R-250 as described by Sambrook et al. (30).

Results

Cytopathology and polyhedron production

In order to determine if the Md203 cell line differed in the susceptibility to ManeNPV, cells were monitored for cytopathic effects (CPEs) and polyhedral inclusion body (PIB) formation. Light microscopy studies shown in Figure 1, summarizes the infectivity of ManeNPV to Md203 cell line. The mock-infected cells appeared normal throughout the incubation period. Typical NPV cytopathic effects were observed in virus infected cells. Infection with ManeNPV showed pronounced CPEs in which the nuclei of the cells were a little swollen and the nuclear membranes were hypertrophied to the cell membranes and impairment in cell proliferation, by 24 h p.i. At 36 h p.i., the first PIB formation was observed clearly in a few Md203 cells (Figure 1). While cells were generally granulated and rounded and developed nuclear hypertrophy, some cells lost their general shape by extendings. At 48 h p.i., sizes of PIBs increased, cells began to darken, and approximately 60-65% of total cells had numerous PIBs. At 72 h p.i., darkening increased and the level of PIB-including cells reached 95%. Also, at 96 h p.i., all the infected cells contained many polyhedra and some of the cells were beginning to detach from the bottom of the flasks. As the cells became more hypertrophied and some of the cells lyzed, PIBs in nuclei released into the culture media.

Budded virus production

Culture media was harvested from the ManeNPV-infected Md203 cells at 0, 24, 48, 72 and 96 h p.i., and the yield of budded virions in the culture media was titred by the 50% tissue culture infectious dose ($TCID_{50}$) using the same cell line. The results showed that ManeNPV replicated in Md203 cell line and released BVs at different amounts at different times into the culture medium (Figure 2). At 0 h p.i., the

basal virus titer was determined approximately 1 X 10^4 p.f.u./ml. The infectious virus yield at 24 h p.i. was 1.73 X 10^5 p.f.u./ml. The number of BV's in other samples were 5.49 X 10^5 p.f.u./ml, 4.78 X 10^6 p.f.u./ml and 1.19 X 10^7 p.f.u./ml at 48, 72 and 96 h p.i., respectively. Significant increase in BV titer was observed at 96 h p.i. as 10^3-fold.

Viral DNA replication

To determine whether ManeNPV replicated in Md203 cell line, viral DNA replication in infected cells were investigated by slot blot hybridization. Viral DNA was extracted from infected cells at 0, 2, 4, 6, 8, 10, 12, 24, 48 and 72 h p.i., blotted onto a transfer

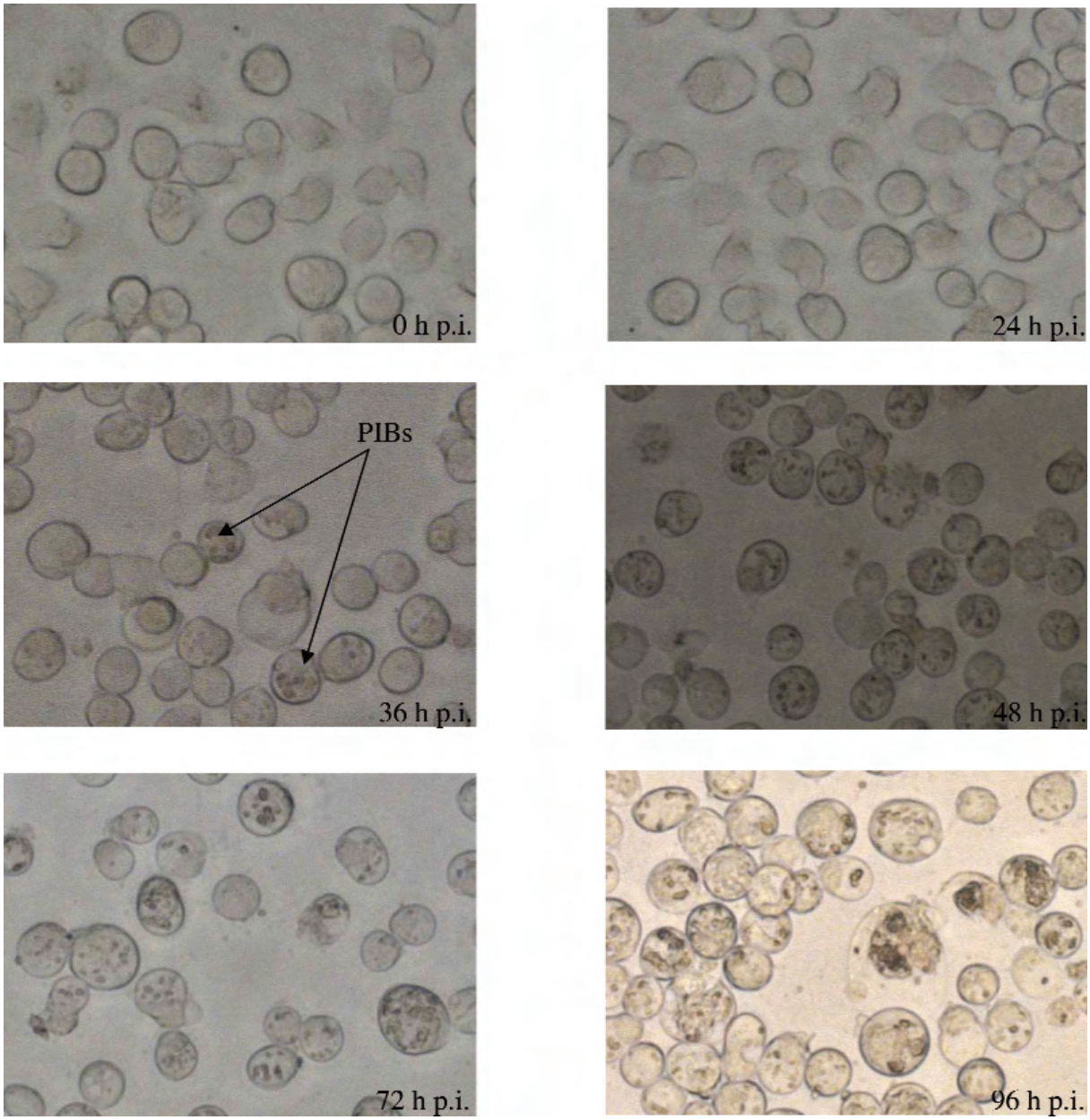

Figure 1. Cytopathology and polyhedral formation in ManeNPV infected-Md203 cell line. Cells (1×10^6 cells/well in 6-well) were infected with ManeNPV-pp at an input MOI of 10 pfu/cell and incubated at 27 °C. Infected cells were observed at 12 h intervals and photographed at 24 h intervals 96 h p.i. PIB: Polyhedral inclusion body.

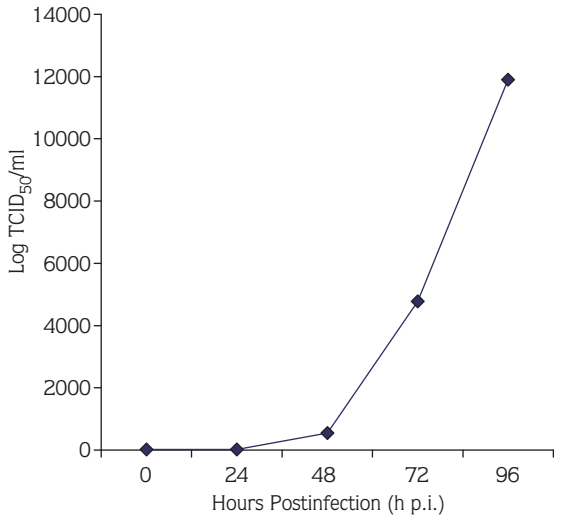

Figure 2. Curve of endpoint dilution titers in ManeNPV-infected Md203 cell lines. Cell culture media was collected at various times postinfection and titrated by $TCID_{50}$ assays on Md203 cells. The numbers of budded is $TCID_{50} \times 10^4$.

membrane, and hybridized with labeled ManeNPV DNA as described under Materials and Methods. In ManeNPV-infected Md203 cells, viral DNA replication was initiated before 24 h p.i. and increased between 48 and 72 h p.i. The rate of synthesis reached at highst level at 72 h p.i. (Figure 3). Mock infection was used as a control and did not hybridzed with any probe.

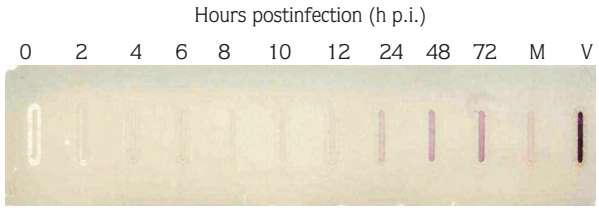

Figure 3. Slot-blot analysis of viral DNA in Md203 cells infected with ManeNPV. Cells (1×10^6 cells/well in 6-well) were infected with ManeNPV-pp at an input MOI of 10 pfu/cell. At 0, 2, 4, 6, 8, 10, 12, 24, 48 and 72 h p.i., infected cells were lysed by adding 800 ml of 0.5 N NaOH and the suspension was neutralized with 80 ml of 10 M NH_4-acetate. Cell lysates (100 ml for each time) were blotted on to transfer membrane, hybridized with labelled ManeNPV *polh* DNA (prob), and detected using Detection Starter Kit I (Roche Diagnostics) as described by manufacturer. M: Mock-infected cells lysate. V: ManeNPV viral DNA.

Viral protein synthesis

Experiments were conducted to examine if polyhedrin was synthesized in ManeNPV-infected Md203 cell lines. At 0, 12, 24, 36, 48, 60, 72, 84 and 96 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 Md203 cell line before 36 h p.i. (Figure 4). It was observed as a dense band at later times.

Discussion

Biological characterization of a baculovirus isolate is an essential preliminary step for a successful utilization and development of any biological control and biotechnological agent. Until now, a lot of clones have been developed to use as microbial control

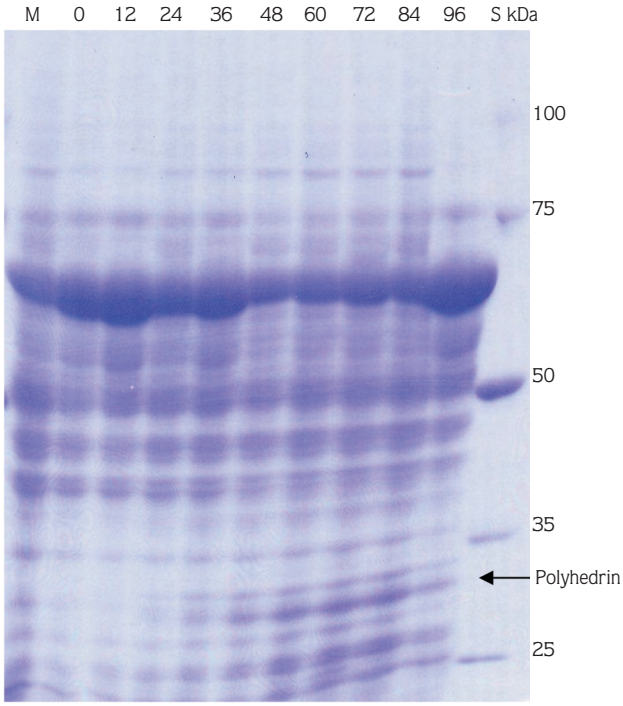

Figure 4. SDS-PAGE analysis of viral polypeptides and polyhedrin in Md203 cells. Infected cells were harvested at 0, 12, 24, 36, 48, 60, 72, 84 and 96 h p.i., and lyzed. Polypeptides in the lyzed were resolved on 12% SDS-polyacrylamide gel and stained with Comassie brillent blue. M: Mock-infected cells, S: Standart molecular masses.

agents and gene expression vectors. Improving a new and more effective pest control agent and expression vector is possible with the isolation and determinetion *in vitro* replication properties of new baculoviruses. However, the underlying mechanism(s) by which virulence is increased has not yet been elucidated. So in this study, replication properties of a baculovirus from *Malacosoma neustria*, ManeNPV, was determined in cell culture system to improve different strategies about its biotechnological applications.

The susceptibility of *Malacosoma disstria* cell line, Md203, to ManeNPV was studied on the basis of CPE and PIB formation, BV production, viral DNA replication, and viral structural protein synthesis. During infection, CPEs were similar to those of other NPV replication studies (15,25,31,32). Typical characteristics of baculovirus productive infections displayed granulation, swollen, nuclear hypertrophy, the presence of inclusion bodies, and nuclear darkening (Figure 1) (33). First CPEs were determined before 24 h p.i., and first PIB formation was detected clearly at 36 h p.i. At 96 h p.i., it was determined that all of the cells were clearly moribund, and had different size and amounts of PIBs. While uninfected cells were active, squamous and proliferate, infected cells were round and some cells appeared to contain higly refractive bodies within them which resembled PIBs. The susceptibility of lepidopteran cell lines to baculoviruses are different. CPEs and PIBs appeared at different time in various infections. PIBs yields was significantly changed from cell line to cell line and from virus to virus. PIB productions may occurre between 24 h p.i. and 72 h p.i. in different cell lines (25,31,32). Erlandson et al. (34) infected three cell lines from *M. disstria* haemocytes with plaque-purified MadiNPVs from *M. disstria*, and PIBs were determined at 72 h p.i. in these infections. In this study, we determined that PIBs were produced by 36 h p.i.

The results of CPEs were supported by the production of infectious virus yields. At 0 h p.i., the basal virus titer was determineted approximately 1 X 10^4 p.f.u./ml. The significant increase in the amount of BV occurred at 48 h p.i., and BV production continued to produce at 72 and 96 h p.i. Consequently, titer become 1.19 X 10^7 p.f.u/ml at 96 h p.i., this showed 10^3-fold increase.

The course of virus replication showed typical baculovirus single-step growth. Growth patern appeared to continue increasing even after 96 h p.i. It is known that BV productions in various baculovirus infected-cells could be various from productive infections to non productive (15,16). In a previous study, Shirata et al. (35) reported that increase of baculovirus BV titters in different cell lines changed about 6.6-fold to 15.000-fold. In another study, Demir and Demirbağ (17) showed that BV titters were increased 10^5-fold in both *Hyphantria cunea* nucleopolyhedrovirus infected Sf21 and LdElta cell lines at 72 h p.i. Huang et al. (36) showed that Md210, a cell line from *Malacosoma disstria* did not support production of viable *Spodoptera littoralis* nucleopolyhedrovirus progeny. Erlandson et al. (34) reported that three cell lines derived from *Malacosoma disstria* haemocytes, UA-Md203, 210 and 221, were permissive for plaque purified Malacosoma disstria NPV (MadiNPV). However, each of the cell lines had different susceptibility to MadiNPV strains and maximal BVs became at 48 h p.i., and not increasing BV production following times. However, in this study, maximal BV production was detected at 96 h p.i.. While BV production is low in ManeNPV-infected Md203 cell line system, yield in this system is as high as in MadiNPV-infected Md203 cell line system. This suggested that Md203 cells are also susceptible to ManeNPV infection, and it is productive to ManeNPV similar to MadiNPV.

Slot-blot hybridization data showed that DNA replicated efficiently in Md203 cells by 24 h p.i., and the maximal rate of viral DNA synthesis was between 72 to 96 h p.i. DNA replication of baculoviruses occurred generally at 8-10 h p.i., and detected at approximetly 10-12 h p.i. by slot-blot hybridization (35). Even some baculoviruses DNAs can replicate at 4 h p.i. in defferent cell lines. A previous study reported that AcNPV DNA synthesis in Ld652Y cells 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. (37). Another study also indicated that HycuNPV DNA replication in Sf21 and LdElta cell lines started at approximately 12 h p.i., reached at high leves by 24 h p.i, and the rate of synthesis was maximal between 24 and 48 h p.i. (17). It is known that DNA replication was also initiated at 18-20 h p.i. in some

experiments (38). In another study, the course of DNA replication of *Helicoverpa armigera* single nucleupolyhedrovirus in Hz-AM1 cells from *Helicoverpa zea* Rikvin et al. (39), showed that viral DNA replication was detected first at 24 h p.i. (as in this study), DNA steady state levels increased significantly from 24 to 48 h and sometimes from 48 to 96 h p.i. While *Spodoptera littoralis* nucleopolyhedrovirus DNA was detected in Md203 cells derived from *Malacosoma disstria* within 4 h p.i., Md203 displayed a pattern of time-dependant increase in the amount of SpliNPV DNA. In Md203 cells, the intensity of SpliNPV DNA hybridization decreased consistently from 4 h p.i. to 32 h p.i. suggesting that SpliNPV did not replicate in these cells. The previously reported results, comparable to the results of this study, determined that ManeNPV DNA replication in ManeNPV-infected Md203 cell system was approximately similar to generel baculovirus DNA replication.

The results of SDS-PAGE showed that viral polyhedrin protein synthesis occurred in infected cells before 36 h p.i., and reached at higher leves at 48 h p.i., 72 h p.i. and 96 h p.i. Polyhedrin is the product from a very late gene and a polypeptide of about 28 kDa. Its expression is dependent upon the transactivation by the products from very late expression factor-1 gene and some of the late expression factor genes including *ie-1* and *lef-2* (40). These results are similar to a productive infection study reported by Liu and Bilimoria (41).

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 which 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.

Infection steps of ManeNPV in Md203 cells were successfully completed as *in vitro*. The results and observations strongly indicate that *Malacosoma neustria* nucleopolyhedrovirus can normally multiply in *Malacosoma disstria* cells and the pattern of ManeNPV multiplication is similar to other NPVs. The ability of the Md203 cells to support efficient replication of ManeNPV allowed to study biological characterization of ManeNPV in cell culture system. This is the first report on diteal investigation of replicational properties of *Malacosoma neustria* nucleopolyhedrovirus in cell culture system. Replicational charaterization of this novel ManNPV isolate is a prerequisite to fully determine its potential as a biological control agent and to improve its insecticidal activity through genetic manipulation.

Thus, the present study developed a new NPV-cell culture system which exhibited distinct interactions between NPV and host cell. This NPV-cell culture 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, and improving this virus as biological control agent and gene expression vector.

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