Characterization of the infection cycle of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus in *Lymantria dispar* cells

Mary B. Bradford, Gary W. Blissard and George F. Rohrmann*

Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331, U.S.A.

To characterize the infection cycle of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus in *Lymantria dispar* cells, the time course of DNA synthesis and polyhedron production, and the onset and rate of budded virus production were investigated at three different m.o.i. (5, 10 and 100). In addition, the time course of expression of three proteins (gp64, p39 and polyhedrin) representative of three temporal classes of baculovirus genes was also analysed using Western blot analysis. DNA synthesis began at 12 to 18 h post-infection (p.i.). The rate of budded virus (BV) production reached maximal levels at 24 to 36 h p.i. and continued at high levels indicating that BV production was not turned off late in infection. Polyhedra were first observed at 48 h p.i. The m.o.i. appeared to influence the magnitude but not timing of early events in the viral infection cycle (gp64 expression and DNA synthesis) and also influenced the initial levels of BV production and the percentage of cells containing occlusion bodies. The m.o.i. had little influence on the final rates of BV production and the time of detection of p39 and polyhedrin on Western blots.

Introduction

Nuclear polyhedrosis viruses (NPVs) are a subgroup of the Baculoviridae which are a family of viruses with large double-stranded supercoiled DNA genomes of 88 to 150 kb that are pathogenic for insects. They are characterized by a complex infection cycle that produces two structurally distinct virion phenotypes (for a review, see Blissard & Rohrmann, 1990). The polyhedra-derived virus (PDV) phenotype contains nucleocapsids occluded within crystalline occlusion bodies (termed polyhedra) composed of the 29K polyhedrin protein. The occlusion bodies, which are stable in the environment, serve to spread the virus between insects. By contrast, the budded virus (BV) phenotype buds from infected cells into the insect haemocoel and serves to spread the infection from cell to cell within insects. A major structural difference between PDV and BV virions is the origin of their envelopes. The BV phenotype acquires its envelope by budding through the plasma membrane of infected cells which have been modified by the insertion of a virus-encoded glycoprotein, gp64. The PDV envelope is formed de novo in the nucleus where PDVs are subsequently occluded. In infected cells, the expression of baculovirus genes is believed to occur in a cascade of events in which each successive phase is dependent on the previous phase. There are two general phases, an early phase which precedes viral DNA replication and a late phase that occurs during and after viral DNA replication. The early phase is subdivided into immediate early and delayed early stages. Immediate early genes are those genes which can be transcribed by uninfected insect cells and require no viral gene products. Delayed early genes are expressed before DNA replication but require viral gene products for their transcriptional activation. Late genes are transcribed after or along with the onset of viral DNA synthesis and are transcribed from the late baculovirus A/GTAAG promoter. Hyperexpressed late (or very late) genes are distinguished from other late genes by the fact that mRNAs from most late genes decline at very late times post-infection (p.i.), whereas mRNA levels of hyperexpressed late genes remain high throughout the infection cycle. Numerous late genes have been characterized, but only two, those encoding polyhedrin and p10, are hyperexpressed.

The multicapsid NPV of *Autographa california* (AcMNPV) is the best characterized baculovirus and the major events of its replication cycle have been extensively studied. In this system immediate early genes are expressed by 0 to 1 h p.i. (Guarino & Summers, 1987; Chisholm & Henner, 1988) followed by DNA synthesis at 6 h p.i. (Tjia et al., 1979; Erlandson & Carstens, 1983). Budded virus production occurs by 12 h p.i. and occluded virus particles are observed at about 12 to 14 h p.i. (Volkman et al., 1976). Although AcMNPV is familiar to many investigators because it is commonly used as an
expression vector, over 600 other baculoviruses have been identified, many of which appear to have significantly different replicative and biological properties from AcMNPV. One of these, the MNPV of *Orgyia pseudotsugata* (OpMNPV), demonstrates a collinear genome organization with AcMNPV (Leisy *et al.*, 1984). However, it shows an amino acid sequence similarity of specific genes as low as 26% and may lack at least two genes present in AcMNPV (Gombart *et al.*, 1989). In addition to these differences, the OpMNPV has a different host range and in the laboratory replicates in a *Lymantria dispar* cell line.

In this report we describe the time of the major events of OpMNPV replication in *L. dispar* cells. In addition to characterizing the time course of DNA replication and budded and occluded virus production, we used Western blot analysis to investigate the expression of three major structural proteins which are representative of different temporal gene classes. These include gp64 which is the major envelope glycoprotein of BV and is expressed both as an early and a late gene product (Blissard *et al.*, 1989; Whitford *et al.*, 1989), p39 which is a capsid protein and is expressed from a late gene (Pearson *et al.*, 1988; Blissard *et al.*, 1989; Thiem & Miller, 1989) and polyhedrin which is encoded by a hyperexpressed late gene (Rohrmann, 1986). These analyses were done at three m.o.i. (5, 10 and 100) to determine the influence of m.o.i. on the time course of infection.

**Methods**

**Insect cell lines, virus, infections and antibodies.** *L. dispar* cell line LD652Y was cultured in TNM-FH medium supplemented with 10% foetal bovine seire (Summers & Smith, 1987) at 27 °C. The clonal isolate of OpMNPV used in these studies was described by Quant-Russell *et al.* (1987). The p39 monoclonal antibody (Mab) (236) and polyhedrin polyclonal antibody were described in Pearson *et al.* (1988) and Rohrmann (1977), respectively. The gp64 Mab (AcV,) was described by Hohmann & Faulkner (1983) and applied to OpMNPV as described by Blissard & Rohrmann (1989). For all m.o.i. studies, cells were seeded at 4 x 10⁵ cells/ml in 24-well microtitre plates and virus stocks were added at the desired m.o.i. (5, 10 or 100). After a 1 h adsorption period, the medium (1 ml) was removed and cells were rinsed with 1 ml of fresh medium then replaced with another 1 ml of fresh medium.

**Measurement of viral DNA in infected cells.** To measure the relative quantities of viral DNA in infected cells, supernatants were removed from triplicate samples for each time point and the cells were disrupted and RNA removed by adding 400 µl of 0.5 M-NaOH. Ten ng of plasmid DNA (pBS) was added to each sample as an internal standard (see below). After boiling for 3 min, ammonium acetate was added to a final concentration of 1 M and samples were stored at -80 °C. Each sample was brought to 500 µl with H₂O and the aqueous solution was extracted sequentially with equal volumes of phenol, phenol/chloroform (1:1) and chloroform/isooamyl alcohol (24:1). After ethanol precipitation, DNA samples were resuspended in 100 µl H₂O. To denature the DNA, 100 µl of 0.6 M-NaOH was added to each DNA sample and incubated at 60 °C for 5 min. Samples were then brought to 1 ml in ammonium acetate in a final volume of 1 ml. DNA from 2 x 10⁶ cells equivalents was added to each slot of a slot blot apparatus (Schleicher & Schuell) and bound onto GeneScreenPlus hybridization membranes (New England Nuclear) as described by Kafatos *et al.* (1979). OpMNPV genomic DNA was labelled by the hexamer labelling procedure (Feinberg & Vogelstein, 1983) to a specific activity of approximately 9 x 10⁸ c.p.m./µg and added to slots at a concentration of 2 x 10⁶ c.p.m./ml of hybridization buffer (7 ml/slot). Blots were hybridized for approximately 14 h under stringent conditions (50% formamide, 42 °C) (Maniatis *et al.*, 1982) and then washed as follows: five times for 10 min in each wash of 2 x SSC, 0.1% SDS at room temperature; three times for 20 min in each wash of 0.1 x SSC, 0.1% SDS at 60 °C. Radiolabelled DNA hybridizing to the slots was visualized and quantified using an AMBIS Radioanalytic Imaging System (Automated Microbiology Systems). To correct for variability in extraction and precipitation steps, the blots were stripped of the labelled probe DNA by incubating with 0.4 M-NaOH, then hybridized with a 32P-labelled pBS plasmid DNA and processed and analysed as described above. The lowest value from the pBS hybridization to the internal standard (10 ng pBS DNA) was assigned a value of 1.0. Deviation of each sample from the average was calculated and data from OpMNPV hybridizations were subsequently adjusted relative to internal standards.

**Measurement of BV production.** The rate of BV production was quantified by measuring the TCID₅₀ produced over defined time periods. For each m.o.i., three sets of wells (4 x 10⁵ cells/well) were infected and at each time p.i., the supernatants were completely removed from each set of wells for each m.o.i. and replaced with 50% conditioned medium (conditioned TNM-FH medium was produced by mixing a 1:1 ratio of fresh TNM-FH with TNM-FH medium which had been conditioned by incubation on uninfected *L. dispar* cells for 24 h, removed and stored at 4 °C until use). This procedure was repeated on each m.o.i. for each time point analysed.

To determine the TCID₅₀ produced over each time period, cell suspensions (3 x 10⁵ cells/ml) were seeded into 96-well microtitre plates (50 µl/well) and an equal volume (50 µl) of each viral supernatant dilution was added per well. This procedure was done in triplicate for each of the three wells for each time point. After incubation for 5 to 7 days at 27 °C, plates were scored for infection and then the entire supernatant (100 µl) from each well was added to freshly seeded wells containing 50 µl fresh cells (3 x 10⁵ cells/ml). This procedure allowed the amplification of infectious virus present at low levels in the first plate and thus increased the sensitivity of the assay. After 5 to 7 days, the second plate was scored and the TCID₅₀ was calculated from the three sets of data for each well processed from each m.o.i. by endpoint dilution analysis (Reed & Muench, 1938; Summers & Smith, 1987). This resulted in triplicate TCID₅₀ values for each m.o.i. at each time point.

**Western blots.** To determine the time course of expression of specific proteins, infected cell proteins were analysed by Western blot analysis at various times p.i. For isolation of infected cell proteins, each well of a 24-well plate (Falcon) was seeded with 1 ml of a cell suspension (4 x 10⁵ cells/ml). After allowing the cells to attach (1 to 6 h), the medium was removed and virus was added at an m.o.i. of 5, 10 or 100 in 150 µl TNM-FH. After a 1 h adsorption period, the virus was removed and the cells were rinsed with 1 ml of TNM-FH and replaced with 1 ml of TNM-FH. At specified times p.i., total cells and medium from wells were collected and centrifuged (5000 r.p.m., 2 to 3 min). The following protocols were used for preparing samples for characterization of each specific protein.

(i) gp64. Cell pellets were suspended at 8 x 10⁶ cells/ml in 50 µl phosphate-buffered saline (PBS) (PBS is 120 mM-NaCl, 2.7 mM-KCl in 10 mM-phosphate buffer pH 7.4) containing 0.5 mM-FMSF and stored...
frozen at -80 °C. Five µl of 10 x DNase buffer (500 mM-Tris, 50 mM-MgCl2 pH 7.5) and 1.25 µg of DNase were added to 50 µl of thawed cells and incubated for 15 min at 37 °C. An equal volume of 2 x SDS-PAGE sample buffer (Laemmli, 1970) was added and the mixture was boiled for 3 min. An amount equivalent to 6.2 x 10⁴ cells (15 to 19 µl) was loaded in each well of a 12% SDS-polyacrylamide gel. Samples used for the detection of p39 and polyhedrin were treated with NaOH to dissolve polyhedra and ensure the complete solubility of all proteins.

(ii) p39. Cell pellets were resuspended in 50 µl PBS, 0.5 mM-PMSF to a concentration of 8 x 10⁶ cells/ml, and NaOH was added to a final concentration of 0.1 M and incubated at 60 °C for 10 min. An equal volume of 2 x SDS-PAGE sample buffer was added and treated as described above.

(iii) Polyhedrin. Cell pellets from two wells were resuspended in 94.5 µl Grace’s medium (Gibco) containing 0.5 mM-PMSF and 5 µl 1 M-NaOH resulting in a final cell concentration of 8 x 10⁶ cells/ml. An equal volume of 2 x SDS-PAGE sample buffer was added and treated as above.

For Western blot analysis of proteins, SDS-PAGE gels were electroblotted onto nitrocellulose membranes, and reacted with the appropriate antibodies as described previously (Quant-Russell et al., 1987). Antisera were used at the following dilutions: p39 MAb 236 ascites fluid, 1:100; polyhedrin polyclonal antiserum, 1:400; gp64 AcV5 MAb tissue culture supernatant, undiluted.

**Results**

**Time course of viral DNA replication**

To investigate viral DNA replication, ³²P-labelled OpMNPV DNA was hybridized to slot blots of total DNA from L. dispar cells infected at three different m.o.i. (5, 10 and 100) (Fig. 1). The relative amount of virus-specific DNA increased dramatically between 12 and 18 h p.i. for cells infected at m.o.i. 100. Cells infected at m.o.i. 5 and 10 showed a more gradual DNA increase starting between 12 and 18 h p.i. In two separate sets of experiments done with the three m.o.i. analysed in triplicate, the values for relative DNA concentration showed small standard deviations for each m.o.i. for samples taken up to 18 h p.i. (Fig. 1). However for time points at 24 h p.i. and thereafter, the values for m.o.i. 10 and 100 showed large overlapping standard deviations. The reason for this variation in the standard deviation at later time points is not clear but suggests that retention of viral DNA in the cells late in infection is highly variable between experimental wells.

**Time course of BV and occlusion body production**

To determine the rate of BV production, supernatants were completely removed from infected cultures and replaced with 50% conditioned medium at specific times p.i. and the TCID₅₀ was determined for each supernatant (Fig. 2). This allowed determination of the amount

![Fig. 1. Time course of accumulation of viral DNA in OpMNPV-infected L. dispar cells infected with OpMNPV at m.o.i. 5 (●), 10 (■) and 100 (○). The relative quantities of DNA at various times p.i. were determined by hybridization of a 3²P-labelled OpMNPV DNA probe to slot blots of infected cell DNA isolated from equivalent numbers of cells infected at various m.o.i. Each c.p.m. value was determined from three replicate blots and was read directly using an AMBIS Radioanalytic Imaging System. One standard deviation is indicated.](image)

![Fig. 2. Rate of BV production in cells infected at three m.o.i. Cells were infected at m.o.i. of 5 (●), 10 (■) and 100 (○) and at various times p.i. cell culture supernatants were completely removed and the TCID₅₀ was determined as described in Methods. The medium was replaced with 50% conditioned medium (from uninfected cells) at each time point. The data up to 72 h p.i. were determined by titrating supernatants which were incubated with infected cells for 12 h. The titres for the final two time points (96 and 120 h) were determined from supernatants incubated for 24 h intervals and the values were divided by two to yield the average BV production for 12 h. One standard deviation is indicated. Where the standard deviations overlap for different m.o.i., the standard deviation encompassing all the points is indicated.](image)
of BV (TCID$_{50}$) produced between time points. These titres indicated that BV is produced at a maximum rate between 24 and 48 h p.i. for m.o.i. 100 ($8.7 \times 10^8$ TCID$_{50}$/12 h). Infections with an m.o.i. of 5 produced BV at a maximum rate (> $10^8$ TCID$_{50}$/12 h) from 24 to 96 h p.i. An m.o.i. of 10 also resulted in high rates of production throughout the late time periods. Although there was a 1 to 1.4 log$_{10}$ decrease in the rate of TCID$_{50}$ production between the maximal rate at 24 to 48 h p.i. and the final rate at 96 to 120 h p.i., these results indicate that BV production is reduced but not turned off late in the infection. The onset of BV production for all three m.o.i. occurred between 12 to 24 h with the rate of production reaching a plateau by 36 h p.i. The onset of BV production appears to follow closely the initiation of DNA synthesis for all three m.o.i.

The percentage of cells which contained polyhedra in their nuclei was also examined (Fig. 3). Polyhedra were detected at 48 h p.i. and continued to accumulate throughout the late infection period. In cells infected at an m.o.i. of 100, polyhedra were observed in 35% of the cells very late in infection. However in infections using m.o.i. 5 and 10, polyhedra were observed in only about 15% of the cells. Occlusion bodies were never found in more than about 35% of the cells even when higher m.o.i. (e.g. m.o.i. 300) were used (data not shown). C.p.e. from virus infection was observed in most cells even when the lower m.o.i. were used (m.o.i. 5 and 10) indicating synchronous infection of most if not all cells. Therefore, the above results suggest an inability of some cells to produce visible polyhedra rather than the lack of synchronous infection.

**Time course of expression of gp64, p39 and polyhedrin**

The time course of expression of three viral proteins was examined by Western blot analysis (Fig. 4). Gp64, a viral envelope glycoprotein, is produced both early and late in infection as the gp64 gene has both early and late promoters (Blissard & Rohrmann, 1989). It was first detected on Western blots at 6 h p.i. for all m.o.i.; however, the amounts of gp64 detected appeared to be m.o.i.-dependent. P39, a capsid protein which is expressed late in infection and accumulates in the infected
cell nucleus by 48 h p.i. (Pearson et al., 1988), was first observed on Western blots of cells infected at all three m.o.i. in the 36 h p.i. samples. By 48 h p.i., abundant quantities of p39 were readily detected in extracts of cells from all three m.o.i. Polyhedrin, a hyperexpressed late gene, was not observed on Western blots until 48 h p.i. in cells infected at all three m.o.i.

Discussion

In this report we have examined major events in the replicative cycle of OpMNPV in L. dispar cells. By Western blot analysis, we detected the product of a gene with an early promoter, gp64, at the earliest time examined (6 h p.i.). As gp64 is the major envelope glycoprotein, it is likely that its early expression allows for its migration and modification of the host cell membrane in preparation for the production of BV. Between 12 and 18 h p.i., DNA replication begins and is consistent with the onset of BV production which occurs between 12 and 24 h p.i. We determined that BV reaches maximal rates of production at 36 to 48 h p.i. and continues at a relatively high rate throughout the infection indicating that both viral phenotypes are produced late in infection. The rate of BV production plateaus or declines somewhat when polyhedra appear (48 h p.i.). P39 protein was not observed on Western blots until 36 h p.i. It is likely that it is synthesized earlier [we have observed it at low levels at 24 to 28 h p.i. using another Western blotting protocol (Pearson et al., 1988)] but its relative concentration is probably reduced at earlier times because it is removed from the cells by virus budding. Polyhedra were not observed until 48 h p.i. and this corresponds with the appearance of polyhedrin on Western blots at 48 h p.i. (Fig. 4). After 48 h p.i., polyhedra were observed in an increasing percentage of infected cell nuclei.

The m.o.i. appears to influence the level of expression of genes with early promoters. Although gp64 was observed on Western blots by 6 h p.i. with all m.o.i., its concentration appeared to be higher when cells were infected at m.o.i. of 10 and 100 (Fig. 4). Such differences probably result from a gene dosage effect. Samples with an m.o.i. of 100 also demonstrated a more rapid accumulation of virus-specific DNA although m.o.i. 10 cells eventually contained similar amounts (Fig. 1). We speculate that the more rapid accumulation of viral DNA in cells infected at high m.o.i. may be caused either by higher initial template numbers, or a higher level of early gene products involved in DNA replication (i.e. DNA polymerase or proliferative cell nuclear antigen; Tomalski et al., 1988; O'Reilly et al., 1989). The onset of late viral phenomena did not appear to be substantially affected by the different m.o.i. The late events that we monitored included the rate of BV production (12 to 24 h p.i.), polyhedra production (36 to 48 h p.i.), p39 synthesis and polyhedrin synthesis. Although the onset of the appearance of polyhedra in infected cell nuclei occurred at similar times, polyhedra appear to accumulate to about twice the level in cells infected at an m.o.i. of 100. This phenomenon has also been reported in L. dispar MNPV (LdMNPV) infecting L. dispar cells (McClintock et al., 1986) and may be due to the higher levels of DNA synthesis early in infection resulting in more DNA being available for virus and thus polyhedral inclusion body (PIB) production.

In a similar investigation McClintock et al. (1986) infected L. dispar cells with LdMNPV at an m.o.i. of 100 and found a rapid increase of BV (TCID₅₀) produced at 24 h p.i. which was similar to our observations for OpMNPV. In addition, PIBs were first observed at 48 h p.i. similar to the OpMNPV system. By contrast, the replicative cycle of the AcMNPV virus in either Spodoptera frugiperda or Trichoplusia ni cells appears to occur at a more rapid rate than either the OpMNPV or LdMNPV system. In one study in which cells were infected at m.o.i. 100, viral DNA replication was observed to begin at 6 h p.i. (Tjia et al., 1979). In another study, BV production in AcMNPV-infected T. ni cells began at 7 to 10 h p.i. (Volkman et al., 1976).

The variability in replicative cycle times could be dependent on features of either the virus, the host cell, or both. In the AcMNPV the homologous repeat sequences (hrs) appear to act as enhancers of early gene expression (Guarino & Summers, 1986). It is possible that the presence of such enhancers in AcMNPV might be responsible for accelerating early viral synthetic events leading to the more rapid onset of viral DNA replication. OpMNPV may not have enhancers as powerful as those present in AcMNPV and therefore may replicate more slowly.

We thank Dr Margot Pearson, Christian Gross and Becky Russell for their valuable technical advice throughout the duration of this project. This project was supported by grants from the NIH (AI 21983 and F32 AI 07886). This is Technical Report No. 9245 from the Oregon State University Agricultural Experiment Station.

References


(Received 28 May 1990; Accepted 14 August 1990)