Virus morphogenesis of *Helicoverpa armigera* nucleopolyhedrovirus in *Helicoverpa zea* serum-free suspension culture

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*Helicoverpa armigera* single nucleopolyhedrovirus (HaSNPV) replication in *Helicoverpa zea* serum-free suspension culture was studied in detail and the sequence of virus morphogenesis was determined by transmission electron microscopy. By 16 h post-infection (p.i.), virus replication was observed in the virogenic stroma by the appearance of nucleocapsids. Polyhedron formation was detected by 24 h p.i. and the polyhedron envelope (PE) was completely formed by 72 h p.i. PE morphogenesis of HaSNPV is significantly different compared to the extensively studied *Autograph californica* (Ac)MNPV. In AcMNPV-infected cells, fibrillar structures are found in both cytoplasm and nuclei, and the fibrillar structures in nuclei are in close association with maturing polyhedra during PE formation. Fibrillar structures that resemble the AcMNPV fibrillar structures were detected only in the cytoplasm of HaSNPV-infected cells and appeared to interact with calyx precursors there, but their role in PE formation is unclear. However, prominent calyx precursor structures of various shapes and sizes were observed in the nuclei of HaSNPV-infected cells as well, and they appeared to interact with polyhedra during PE formation. Both the calyx precursor structure and the cytoplasmic fibrillar structure were detected only after HaSNPV virion occlusion had started, indicating that they might have a role in formation of PE. Similar calyx precursor structures and cytoplasmic fibrillar structures were observed in both serum-supplemented and serum-free suspension cultures, as well as in HaSNPV-infected larval tissues, indicating that the structures observed are not cell culture artefacts.

**Introduction**

Insect pest control is one of the most important issues facing the 21st Century. *Helicoverpa* and *Heliothis* spp. (commonly referred to as heliothis) are a major focus of pest control programmes throughout the world. They attack well over 60 crops (Christian, 1994). Use of chemical insecticides to control these pests has failed in South-East Asia and Australia, resulting in a major crisis with increasing and more widespread resistance (White *et al*., 1996). Increasing resistance of heliothis to existing chemical insecticides has also increased the cost of control and loss of production (Fitt, 1994; McGahan *et al*., 1991). Failure to control heliothis with chemical insecticides has hastened the development of technology for production of biological pesticides that have the potential to control heliothis effectively.

*Helicoverpa armigera* single nucleopolyhedrovirus (HaSNPV), a wild-type baculovirus, has the potential for use as a biopesticide for effective control of heliothis. HaSNPV is specific and highly virulent to its host (Hughes *et al*., 1983; Teakle *et al*., 1985). The development of in *vitro* production of HaSNPV is becoming increasingly important as the demand for effective heliothis control increases.

Various factors relating to the replication of HaSNPV, such as virus infection kinetics, optimal time of infection (TOI), nutrient consumption and passage effect, have been studied previously (Chakraborty *et al*., 1995, 1996, 1999; Chakraborty & Reid, 1999). These studies were aimed primarily at optimizing production processes for this virus. Despite all the reported studies on HaSNPV, little is known about HaSNPV morphogenesis in *Helicoverpa zea* serum-free suspension culture. In *vitro* production of high quality and biologically active...
HaSNPV necessitates an understanding of virus replication and assembly in this production system. Knowledge of the morphogenic events in this system will aid in optimizing production parameters such as the time of harvest for fully mature polyhedra, and in documenting any structural differences between this system and other reported baculoviruses.

Previous studies on *Helicoverpa zea* SNPV (Goodwin *et al*., 1973; Granados, 1978, 1981; Ignoffo *et al*., 1971; Lenz *et al*., 1991; McIntosh & Ignoffo, 1981; Rice *et al*., 1989; Yamada *et al*., 1982) revealed very little about the morphogenesis of the virus. A brief sequence of morphogenesis was described by Granados *et al*. (1981). However, virion occlusion and polyhedron envelope morphogenesis was not reported in detail. Ultrastructural studies on replication of other NPVs have been performed extensively over the last two decades. Most of these studies were done using either infected larval tissues (Adams *et al*., 1977; Granados & Lawler, 1981; Harrap, 1972b; Hess & Falcon, 1978; Kawamoto *et al*., 1977; Tewari & Datta, 1996), or by using monolayers of cells grown in serum-containing media (Carstens *et al*., 1979; Fraser, 1986; Knudson & Harrap, 1976; MacKinnon *et al*., 1974; Young *et al*., 1993). The most well documented virus morphogenesis process available is for *Autographa californica* NPV (AcMNPV), which was reviewed by Williams & Faulkner (1997).

NPVs have a biphasic replication cycle that is localized in the cell nucleus (Vlak, 1992). The first phase of replication generates nucleocapsids and budded virus. The second phase involves the development of large proteinaceous paracrystalline occlusion bodies, also known as polyhedra, which are composed mainly of a polyhedrin protein of about 30 kDa, in which numerous enveloped virions are embedded. Varying from 0.5 to 15 μm in diameter, the polyhedra stabilize virions, thus allowing them to remain viable for long periods in the environment (Bergold, 1963). Each polyhedron has an electron-dense layer known as the polyhedron calyx or polyhedron envelope (PE), the function of which is unknown. Gross *et al*. (1994) suggested that the PE may prevent virions from dislodging from the polyhedron matrix and, in general, helps seal the virus complex. The PE consists mainly of carbohydrates, with 60% hexose, 29% pentose and small amounts of uronic acids and hexosamines (Minion *et al*., 1979), and at least one protein (Whitt & Manning, 1988).

This paper documents the morphogenic sequence of HaSNPV in *H. zea* serum-free suspension cultures and further discusses prominent structural differences between the morphogenesis of HaSNPV and that of other baculoviruses. This study improves our understanding of HaSNPV replication in serum-free suspension cultures and will undoubtedly aid in the development of an optimized production process of HaSNPV as a commercial biopesticide.

**Methods**

- **Cell line.** *Helicoverpa zea* cells were a gift from CSIRO, Division of Entomology, Canberra, Australia. The cell line was isolated from pupal ovarian tissue, strain BCIRL-HZ-AM1 (McIntosh & Ignoffo, 1981). The uncloned cell line was gradually adapted to SF900II (GIBCO BRL) serum-free medium in suspension culture (Chakraborty *et al*., 1995). Cultures are typically grown in 250 ml Erlenmeyer flasks on an orbital shaker operated at 120 r.p.m., inside a refrigerated incubator at 28 °C.

- **Virus.** HaSNPV (uncloned) passage one stock was established in cells grown in SF900II plus 10% foetal bovine serum (CSL, Australia) with haemolymph collected from larvae (obtained from the Department of Primary Industries, Long Pocket, Queensland). The haemolymph from 22 infected larvae was pooled together into a total volume of 100 ml of medium. It was frozen in liquid nitrogen and later thawed and filtered through a 0.2 µm filter before use. 1.8 ml was used to infect a 20 ml *Helicoverpa zea* suspension culture at 3 × 10^6 cells/ml. 80 ml fresh medium was added at 4 days p.i., to prevent over growth of cells and to provide fresh nutrients. Budded virus of passage one was harvested at 80% cell...
viability (8 days p.i.). The cell suspension was centrifuged at 1000 g for 5 min at room temperature. The cell pellet was discarded and the virus-containing supernatant was stored at -70 °C. Passage two stock was made from passage one virus in the following way, for use in this study. A culture with a 50 ml working volume was seeded at 3 x 10^5 cells/ml in SF900II medium and allowed to grow to 1 x 10^6 cells/ml. It was diluted back to 5 x 10^5 cells/ml with fresh medium and infected with an m.o.i. of 0.5 p.f.u. per cell. Passage two budded virus was harvested at 70% cell viability (5 days p.i.), stored at -70 °C and titred before use.

Quantification of budded virus. The virus titres of passage one and two stocks were determined by a plaque assay using Helicoverpa zea cells in SF900II plus 10% foetal bovine serum cells. 60 mm Petri dishes (Corning) were seeded with 5 ml Helicoverpa zea cells at 3 x 10^5 cells/ml, and incubated overnight at 28 °C. The medium was removed from the cells and 0.5 ml of virus at an appropriate dilution was inoculated onto the plates and rocked gently for 4 h. Virus inoculum was removed before 4 ml of medium–agarose mix was overlaid onto each plate. The overlay consisted of an equal volume of 2 x SF900II plus 10% foetal bovine serum and 2% low melting point SeaPlaque agarose (FMC BioProducts).

Virus infection. Duplicate 50 ml cultures were seeded at 3 x 10^5 cells/ml in SF900II medium and allowed to grow to 1 x 10^6 cells/ml before being diluted to 5 x 10^5 cells/ml with 50 ml fresh medium. These cultures were infected at an m.o.i. of 2 p.f.u. per cell. Samples were collected for transmission electron microscopy (TEM) processing at 4 hourly intervals post-infection for the first 48 h.p.i. and subsequently every 24 h p.i. Samples from mock-infected cultures were also collected and processed for TEM. Cell density and viability were determined daily in triplicate using the 0.1% trypan blue exclusion method (Nielsen et al., 1991). To determine polyhedra density, cells were lysed with 0.5% SDS for 1 h at 28 °C before triplicate counts were done in a haemocytometer counting chamber.

An infected Sf9 cell pellet was obtained from Matthew Rosink (University of Queensland). In brief, Sf9 cells were infected with a recombinant AcMNPV expressing β-galactosidase (pAc-360 β-gal), at an m.o.i. of 0.001 and harvested at 4 days p.i. Another HaSNPV infection in SF900II supplemented with 10% serum was performed to determine that some of the structures observed in the serum-free medium were not a result of the lack of serum. Infections in the serum-supplemented cultures were carried out under conditions similar to those used for serum-free cultures and infected cell samples were collected at 2 and 3 days p.i. for TEM. Fourth instar Helicoverpa armigera larvae were also infected with in vivo-produced HaSNPV polyhedra, and the fat bodies of infected larvae were harvested at 4 days p.i. for TEM.

Transmission electron microscopy. TEM processing was similar to previous reports in the literature but was modified for cell culture samples. The cells were harvested at different times post-infection, pelleted and fixed with 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2, for at least an hour at 4 °C. Cells were washed once with phosphate buffer pH 7.2 at 4 °C and resuspended in 2% SeaPlaque agarose. The cell–agarose block was cut into 1 mm squares and washed twice again with phosphate buffer, before being fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 6.8, for 1 h at 4 °C. The samples were then washed once with 0.1 M cacodylate buffer, pH 6.8, and once with water at room temperature. After washing, they were sequentially dehydrated once in 50, 70 and 90% acetone and twice in 100% acetone for 5 min at each step. Samples were infiltrated with 50% Spurr's low viscosity resin for 1 h, followed by 100% Spurr's resin for a further 1 h. After one change in 100% Spurr's resin, they were left in resin overnight on a rotator. Samples were embedded in fresh resin and polymerized at 60 °C for 3 days. Ultra-thin sections (60–80 nm) were cut with a Leica Ultracut UCT microtome. The sections on copper grids were stained for 2 min in 5% uranyl acetate and 1 min in Reynolds' lead stain before being viewed under a JEOL 1010 transmission electron microscope.

Results

Virus infection

Polyhedra were first detectable at 48 h.p.i. and the yield at this time was 10% of the final peak volumetric yield (Fig. 1). The peak volumetric yield of polyhedra was obtained when cell viabilities were less than 5% (144 h.p.i.). The volumetric yield remained level after 0% cell viability had been achieved, indicating that no disintegration of polyhedra was occurring. The specific yield of polyhedra was 161 ± 3 polyhedra per cell. The size of polyhedra ranged from 0.7 to 1.2 μm, based on measurements of a small number of polyhedra photographed via TEM. These data indicate that the cultures were rapidly infected at an m.o.i. of 2 p.f.u. per cell and achieved a high specific cell yield, thus highlighting that the sequence of virus morphogenesis documented is indeed based on cultures infected under near-optimum conditions.

Sequence of virus morphogenesis

TEM examination of ultra-thin sections of infected cell samples at intervals of 4 h.p.i. allowed determination of the sequence of HaSNPV morphogenesis in Helicoverpa zea suspension cultures. The sequence of infection was documented based on viewing the rapidly infected and high producing cells amongst the population of infected cells.

Mock-infected cells harvested at 24 and 48 h.p.i. were also observed under TEM as a control to the infection studies (Fig. 2 a, b). Infection with an m.o.i. of 2 p.f.u. per cell was insufficient to observe early infection events (such as virus attachment and entry into host cells) in the first 4 h.p.i. Infection with a higher m.o.i. of 10 or 100 p.f.u. per cell could not be performed due to the low virus stock titre (2 x 10^7 p.f.u./ml). Pictures were obtained from the secondary infections (at 24 h.p.i.) to illustrate virus attachment and entry at the plasma membrane (Fig. 2 c). The first indication of virus infection was enlargement of the cell nucleus and formation of a loose network of granular material in the middle of the nucleus (Fig. 2 d). Development of this intranuclear virus replication centre, the virogenic stroma, was first detected at 12 h.p.i. Clumps of heterochromatin, normally seen dispersed throughout the nucleoplasm of an uninfected cell (Fig. 2 a, b), were reduced and displaced peripherally along the inner nuclear membrane by the forming stroma. Maturation of the virogenic stroma yielded a significant and morphologically distinct peristromal compartment of nucleoplasm, called the ring zone (Williams & Faulkner, 1997). Examination of the 16 h.p.i. sample revealed formation of short progeny nucleocapsids in the stromal network (Fig. 2 e). These
Fig. 2. For legend see facing page.
nucleocapsids appeared to associate very closely with the stromal edge. Short membrane profiles were observed in the ring zone. These membrane profiles likely represent the source of virion envelopes. Some unidentified circular structures were also seen near the inner nuclear membrane.

By 20 h p.i., the matured virogenic stroma had condensed into a dense lattice-like structure, filling the nucleus almost completely, and the intrastromal spaces were enriched with nucleocapsids (Fig. 3a). At this time, intranuclear nucleocapsid envelopment was also observed in the ring zone (Fig. 3b). Nucleocapsids formed within the virogenic stroma had moved to the ring zone, specifically to the locale of the membrane profiles, presumably to obtain their envelopes. At 24 h p.i., many virus particles were budding from the plasma membrane, acquiring a loose-fitting envelope. Unenveloped virus was often detected in the cytoplasm and vacuoles. During this interval, there was also an increase in virus replication and virion envelopment with the nucleus. Further enlargement of the cell nucleus had reduced the ‘cytoplasm space’ between the plasma membrane and the nuclear membrane. The virogenic stroma had also reduced in size, resulting in a wider ring zone (Fig. 3c). Early virion occlusion was detected in the ring zone. Polyhedrin proteins were deposited around the enveloped virions, occluding the virions progressively (Fig. 3d). Only enveloped virions appeared to be occluded. By 28 h p.i., the polyhedra had increased in size, occluding more virions and were approaching maturity. Some electron-dense ring structures were first detected in the virogenic stroma around this time (Fig. 3e). These structures appeared as multiple layers of circles (Fig. 3f).

Fibrillar structures were also noted in the cytoplasm of infected cells (Fig. 4a). The electron-dense ring structures had developed into irregular folds of various sizes that ultrastructurally appeared to be calyx precursors as observed by Williams & Faulkner (1997). These structures were prominent in the virogenic stroma and ring zone by 32 h p.i. They appeared to have moved to the ring zone, in the close vicinity of the maturing polyhedra (Fig. 4b). Many polyhedra had reached their maximal size by 36 h p.i. (Fig. 4c). The calyx precursor structures were not uniform in size or shape (Fig. 4d, e). They were made up of multiple electron-dense layers and appeared either as ‘stacks’, layers of concentric circles or swirls of irregular shape.

Formation of a PE was first observed at around 48 h p.i. The calyx precursor structures appeared to encircle maturing polyhedra (Fig. 5a). PE formed at this interval were not fully matured, as they were not continuous around the polyhedron (Fig. 5b). By 72 h p.i., many polyhedra were fully mature, each containing a complete electron-dense continuous PE (Fig. 5c). Calyx precursor structures were no longer visible in the nuclei that contained mature polyhedra and cell plasma membranes were deformed. By 96 h p.i., most cells observed had deformed or disintegrated cell plasma membranes but the nuclear membranes remained intact (Fig. 5d). All cell nuclei viewed under TEM at 144 h p.i. were lysed, with mature polyhedra being released into the medium.

Fibrillar structures, presumably the homologues of fibrillar structures observed in all AcMNPV infections, were found only in the cytoplasm of HaSNPV-infected cells cultured in SF900II medium (Fig. 6a). No structure similar to the calyx precursor structures or the fibrillar structures was detected in the cytoplasm of the mock-infected cells (Fig. 2a, b). To understand the significance of these observations and to verify that these structures observed in HaSNPV-infected serum-free suspension cultures are not artefacts, virus morphogenesis in other cell-virus systems was studied. A control infection of SF9 cells with a recombinant AcMNPV expressing β-galactosidase (pAc-360 β-gal) clearly showed previously reported fibrillar structures in both the nuclei and cytoplasm of infected cells (Fig. 6b). The fibrillar structures usually had calyx precursors associated with them. HaSNPV infection in Helicoverpa zea cells grown in serum-supplemented medium also showed fibrillar structures, but located only in the cytoplasm of the infected cells (Fig. 6c). Fibrillar structures were again found located only in the cytoplasm of HaSNPV-infected larval fat cells (Fig. 6d).

In an effort to document that the HaSNPV morphogenic events observed are representative of an infected cell population, cells were harvested at 16, 24, 32, 40, 48, 72 and 96 h p.i., and 50–100 infected cells were scored into six categories based upon the most advanced event recognized in each cell. The categories are virogenic stroma formation, nucleocapsid production, virion envelopment, initial virion occlusion, late polyhedron formation and mature polyhedra. From the same population of scored cells, the percentage of infected cells exhibiting calyx precursor structures and fibrillar structures was also determined. Fig. 7 presents the typical morphogenic process of a HaSNPV-infected cell population. At 16 h p.i., all infected cells viewed were either forming the virogenic stroma or producing nucleocapsids; by 96 h p.i. all infected cells showed polyhedron morphogenesis. Fig. 7 clearly indicates

Fig. 2. Mock-infected cells and infected cells. (a) Mock-infected cell at 24 h p.i. (1 × 10⁶ cells/ml). Clumps of heterochromatin were dispersed throughout the nucleus. (b) Mock-infected cell at 48 h p.i. (2 × 10⁶ cells/ml). Note the increase in vacuoles in the cytoplasm. (c) A budded virus with loose fitting envelope (arrow) entering the cell by endocytosis. (d) Early virogenic stroma (12 h p.i.) forming within the infected cell nucleus, displacing clumps of heterochromatin (arrowheads) to the periphery of the nucleus. (e) At 16 h p.i., the virogenic stroma had condensed into a dense structure and progeny nucleocapsids (arrowheads) appeared in the stromal network (see also insert I). Short membrane profiles (arrows) were observed in the ring zone (see also insert III). An unknown viral structure (insert II) also appeared near the inner nuclear membrane. BV, budded virus; cm, cell membrane; vs, virogenic stroma; h, heterochromatin; Nu, nucleus; nm, nuclear membrane; c, cytoplasm; r, ring zone.
Fig. 3. For legend see facing page.
that both calyx precursor structures and fibrillar structures appeared after virion occlusion had started and increased as polyhedron formation and PE morphogenesis increased.

Discussion

This paper presents a detailed electron-microscopy study on the replication of HaSNPV in serum-free suspension Helicoverpa zea cell cultures, allowing further understanding of HaSNPV morphogenesis in vitro. The morphogenesis of HaSNPV is generally similar to previous reports on MNPV such as AcMNPV (Williams & Faulkner, 1997), Trichoplusia ni (Tn)MNPV (MacKinnon et al., 1974) and Spodoptera frugiperda (Sf)MNPV (Knudson & Harrap, 1976), yet significant differences were observed.

In brief, the main morphogenic events are replication of progeny virus, virion envelopment, virion occlusion and PE formation. The first sign of infection is formation of the virogenic stroma, an electron-dense mantle with electron-lucent intrastromal spaces. Nucleocapsids develop adjacent to the stroma and inside the intrastromal spaces, near the edge of the stromal mantle. The virogenic stroma contains discrete DNA- and RNA-containing structures that are related to replication and packaging of the progeny virus (Young et al., 1993). In vitro studies on AcMNPV-infected Sf21 cells have shown that the electron-dense mantle is extremely sensitive to RNase whereas areas adjacent to the intrastromal spaces are sensitive to DNase.

Nucleocapsids made in the virogenic stroma are subsequently enveloped by one of two processes. They are either enveloped during budding through the cell plasma membrane, acquiring a loosely fitting envelope (budded virus), or enveloped in the nucleus through de novo envelope morphogenesis to form preoccluded virions. In this study, both morphogenic events occurred at about the same time post-infection. These two forms of virus differ morphologically and biochemically. Braunagel & Summers (1994) have demonstrated that the protein and lipid composition of the envelopes of budded virus and preoccluded virions is significantly different.

The de novo intranuclear nucleocapsid envelopment process is clearly observed in this study, and is similar to such processes reported for other baculoviruses (Fraser, 1986; Stoltz et al., 1973). At 16 h p.i., short membrane profiles synthesized de novo in the nuclei of infected cells accumulate in areas of the ring zone, and nucleocapsids that migrate out to the ring zone, form preoccluded enveloped virions. These intranuclear membrane profiles were not observed in mock-infected cultures.

The initial stages of polyhedron formation can be detected by deposition of polyhedrin protein between enveloped virions, forming a typical lattice structure between the virions. Naked, unenveloped nucleocapsids are not occluded. The virion envelope may have a possible role as a site for polyhedrin protein polymerization (Wood, 1980). The envelope of occluded virions in the polyhedrin matrix fits snugly around the nucleocapsid, unlike the budded virus envelope. Enlargement of the polyhedra were observed over time, between 24 and 36 h p.i. It is unclear what factors determine the size of polyhedra or why the deposition of polyhedrin protein ceases at a certain stage. Harrap (1972a) suggested that the available pool of polyhedrin protein monomer may be exhausted at this time.

Early PE morphogenesis in HaSNPV appears to be marked by the appearance of calyx precursor structures in the virogenic stroma. These structures appear to move progressively to the ring zone to interact with maturing polyhedra. Ultrastructurally, they appear similar to the calyx precursors that interact with fibrillar structures of AcMNPV (van Oers & Vlak, 1997; Williams & Faulkner, 1997; Williams et al., 1989) and other baculoviruses (Knudson & Harrap, 1976; MacKinnon et al., 1974). However, in HaSNPV, these structures appeared to emerge and develop randomly in the nuclei, especially in the virogenic stroma, and to have no definite form. The calyx precursor structures often encircle the polyhedra during acquisition of the PE. These calyx precursor structures are most likely the PE precursors, as they appear to be involved in the application of the PE to the surface of the polyhedra. Their appearance coincided with polyhedron formation and they were not observed in the nuclei of infected cells after completion of PE formation, indicating that they have a role in PE morphogenesis.

Fibrillar structures enriched in P10 proteins are commonly found in the nuclei and cytoplasm of AcMNPV-infected cells (Lee et al., 1996; Rohrmann, 1992, Williams & Faulkner, 1997). In AcMNPV-infected cell nuclei, these fibrillar structures usually associate with maturing polyhedra during PE morphogenesis. This phenomenon is frequently observed and well documented for other baculovirus infections such as TnMNPV (MacKinnon et al., 1974), SIMNPV (Knudson & Harrap, 1976) and Orgyia pseudotsugata (Op)MNPV (Gross et al., 1994). However, such a prominent fibrillar structure was not observed in the nuclei of HaSNPV-infected cells. Instead, fibrillar

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Fig. 3. Virion envelopment and polyhedron formation. (a, b) By 20 h p.i., the virogenic stroma contained a large quantity of nucleocapsids (arrowheads) and intranuclear nucleocapsid envelopment was observed in the ring zone. A few enveloped virions were seen at this time (arrows). (c) At 24 h p.i., the virogenic stroma had reduced in size and virion occlusion was observed in the ring zone. (d) Enveloped virions were progressively occluded by deposition of polyhedrin protein. (e) By 28 h p.i., polyhedra were increasing in size and approaching maturity. Calyx precursor structures (arrowheads) were first noticed in the virogenic stroma at around this time. (f) The calyx precursor structures had increased in size and numbers by 32 h p.i. (insert). vs, virogenic stroma; pp, polyhedrin protein; p, polyhedron; r, ring zone; nm, nuclear membrane; c, cytoplasm; v, virion.
Fig. 4. Development of PE precursor structures. (a, b) Calyx precursor structures of various sizes and shapes (arrowheads) were prominent throughout the nucleus (36 h p.i.). It is possible that after formation these structures move out from the virogenic stroma to the ring zone of the nucleus. Note the fibrillar structure (f) in the cytoplasm. (c) The polyhedron had reached the maximal size by 36–40 h p.i. (d, e) The location and shape of the calyx precursor structures (arrowheads) were not consistent. nm, nuclear membrane; p, polyhedron; vs, virogenic stroma; c, cytoplasm; f, fibrillar structure; r, ring zone; v, virion.
Virus morphogenesis of *H. armigera* nucleopolyhedrovirus

Fig. 5. Formation of polyhedron envelope. (a) A ‘cage-like’ structure (arrowhead) formed around a polyhedron (48 h.p.i.). (b) The polyhedron envelope was incomplete and not continuous around the polyhedron at this time. (c) By 72 h.p.i., polyhedra were fully matured and each had an electron-dense envelope. (d) Disintegration of the cell plasma membrane after completion of PE formation but the nuclear membrane remained intact. p, polyhedron; PE, polyhedron envelope; nm, nuclear membrane; Nu, nucleus.

structures (presumptive homologues of AcMNPV fibrillar structures), resembling to some extent the AcMNPV fibrillar structures, were clearly detected only in the cytoplasm of HaSNPV-infected cells. Mock-infected cells showed no fibrillar structures, indicating that these cytoplasmic fibrillar structures are associated with HaSNPV infection events. These cytoplasmic fibrillar structures were first detected at around 28–32 h.p.i., just after initial virion occlusion events in the nuclei. Occasionally, calyx precursor-like structures were seen interacting with these cytoplasmic fibrillar structures. It cannot be ruled out that the absence of fibrillar structure in the nuclei of infected cells and their location only in the cytoplasm is an artefact of serum-free cultures. However, HaSNPV infections in serum-supplemented medium produced similar results (appearance of fibrillar structures in the cytoplasm of infected cells but not in the nuclei). This is further confirmed by the observation that *Helicoverpa armigera* larva fat cells infected with HaSNPV also have cytoplasmic fibrillar structures only.

The P10 protein is known to be a structural component of the fibrillar structures, calyx precursors and PE in AcMNPV-infected cells (Lee *et al.*, 1996). P10 is a poorly conserved protein that is hyperexpressed very late in infection.
Fig. 6. Fibrillar structures in different systems. (a) A fibrillar structure was observed in the cytoplasm but not in the nucleus of HaSNPV-infected cells cultured in SF900II medium (2 days p.i.). Calyx precursor-like structures were seen interacting with the fibrillar structure. (b) Typical fibrillar structures formed during a recombinant AcMNPV infection, interacting with calyx precursors. These fibrillar structures were observed in both the nucleus and cytoplasm of infected Sf9 cells. Sf9 cell culture infected with recombinant AcMNPV (pAc-360 β-gal) (4 days p.i.). (c) infected cell cultured in SF900II supplemented with 10% serum (2 days p.i.). Fibrillar structures were again located in the cytoplasm of the infected cell. Calyx precursor structures were observed in the virogenic stroma (arrow). (d) Fat cell of Helicoverpa armigera infected with HaSNPV (4 days p.i.). A fibrillar structure was located in the cytoplasm of the infected cell and calyx precursor structures in the nucleus (arrow).

Nu, nucleus; nm, nuclear membrane; c, cytoplasm; cp, calyx precursor; p, polyhedron; vs, virogenic stroma; f, fibrillar structure.

(Rohrmann, 1992; van Oers & Vlak, 1997) and is involved in disintegration of the nucleus and release of polyhedra late in infection (van Oers et al., 1993). Recently, the first p10 gene from an SNPV, Buzura suppressaria (Busu)NPV, was identified (van Oers et al., 1998). An AcMNPV recombinant expressing the BusuNPV p10 gene (using a p10-negative AcMNPV virus) formed fibrillar structures in the cytoplasm of infected Spodoptera frugiperda cells. The cytoplasmic fibrillar structures resemble those fibrillar structures found in AcMNPV-infected cells. Thus, it may be possible that P10 proteins of SNPV are located differently to that seen in MNPV, such that in SNPV fibrillar structures may be exclusively located in the cytoplasm. P10 proteins could be present in the nuclei of HaSNPV-infected cells but remain undetected, as they do not form fibrillar structures in the nucleus.

NPV replication and assembly in the cell nucleus evidently
Virus morphogenesis of *H. armigera* nucleopolyhedrovirus

**Fig. 7.** Percentage of an infected cell population exhibiting different stages of virus morphogenic events, at 16, 24, 32, 40, 48, 72 and 96 h p.i. Infected cells (50–100) were viewed under TEM and based upon the most advanced event recognized in each cell, they were scored into six categories: VS, virogenic stroma formation; NC, nucleocapsid production; V, virion envelopment; VO, virion occlusion; PF, late polyhedron formation; MP, mature polyhedra. From the same cells that were scored, the percentage of these cells exhibiting calyx precursor structures (CP) and fibrillar structures (F) were also determined and are presented in the figure (continuous and dashed lines).

involves a complex but elegantly controlled synthetic process. This paper clearly documents the unique morphogenesis of calyx precursor structures and cytoplasmic fibrillar structures during the HaSNPV PE morphogenic process, which has not been reported previously in the literature. Calyx precursor structures and the exclusive cytoplasmic location of fibrillar structures could be unique to HaSNPV morphogenesis since they are observed in both serum and serum-free cultures and also in the *in vivo* system. This ultrastructural study provides an outline of a general mode of HaSNPV replication in *Helicoverpa zea* cells, with time-scales for several morphogenic events, which will facilitate further investigations on production of viral proteins specific to PE formation, such as P10 and PE protein. Research to identify the nucleotide sequences and level of expression of these critical viral proteins is under way.

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**References**


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