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Monodon baculovirus from Australia: ultrastructural observations

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ABSTRACT: The cytopathology, virogenesis and replication of monodon baculovirus (MBV) in *Penaeus monodon* from Australia are described. Electron-dense unenveloped nucleocapsids, not previously described for MBV, are shown in the cytoplasm and attached to the nuclear envelope of infected hepatopancreatocytes. These nucleocapsids comprise a missing link in the published literature on the replication cycle of MBV by providing evidence for the means by which the viral genome travels from the plasma membrane of the hepatopancreatocyte to the nucleus. Features similar to those of MBV from other areas, but not previously reported for MBV from Australia include empty capsids attached to the nuclear pore, central filaments in developing capsids, capsids partly filled with nucleic acid, and filaments in subapical envelope expansions. A model for virogenesis and replication is illustrated which takes into account the new observations as well as previously described ultrastructural characteristics of the developing viral particle.

KEY WORDS: Australian MBV · Baculovirus · Viral replication cycle · Electron microscopy

INTRODUCTION

Monodon baculovirus (MBV) infects epithelial cells of the hepatopancreas (HP) and midgut of *Penaeus monodon* and other penaeid shrimp (Johnson & Lightner 1988). Rod-shaped viruses resembling MBV are widely distributed in the Eastern Hemisphere (Lightner 1996). Similar viruses have been reported in Australia in *P. plebejus* (Lester et al. 1987), *P. monodon* and *P. merguiensis* (Doubrovsky et al. 1988) and *Metapenaeus bennettae* (Spann & Lester 1996). MBV does not cause high mortality, but is believed to lead to a decrease in productivity (Fegan et al. 1991, Flegel et al. 1992). A Taiwanese strain of the virus, *P. monodon* Single Nuclear Polyhedrosis Virus (PmSNPV), has been characterised, with its genome shown to comprise a large supercoiled DNA molecule (Mari et al. 1993).

The ultrastructure, cytopathology, and virogenesis of MBV have been studied by Lightner & Redman (1981), Lightner et al. (1983), Johnson & Lightner (1988), Chen et al. (1989), and Vogt (1992) (see Lightner 1996 for a comprehensive list of references). The profile of virogenesis that is inferred from these studies is one of *de novo* synthesis of virions and assembly of occlusion bodies in the nucleus. Little evidence has been presented to date for proposed stages preceding nuclear events. We report visualisation of electron-dense, unenveloped nucleocapsids of MBV in the cytoplasm and attached to the nuclear membrane. These nucleocapsids comprise a missing link in the published literature on the MBV replication cycle, by providing evidence for how the viral genome travels from the plasma membrane to the nucleus. In addition, ultrastuctural features are described for MBV from Australia that are consistent with those reported for MBV from other areas of the world. We present a model for replication and virogenesis of MBV that is consistent with observations reported in this and earlier publications.

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METHODS AND MATERIALS

Production of MBV-infected postlarvae (PLs). Stage PL15 *Penaeus monodon* (Motoh 1979) were infected with MBV as described previously (Vickers et al. 1992). Briefly, PLs (100 l^{-1}) were infected via seawater with an homogenate of MBV-infected PLs collected from a hatchery in Queensland. Infection was monitored using tissue impressions stained with haemotoxylin and eosin (H/E) (Vickers et al. 1993). HPs were dissected out of PLs under a binocular dissecting microscope when levels of MBV infection appeared to be high (30 to 100% of cell profiles containing occlusion bodies [OBs], between 4 and 10 d post-infection [p.i]).

Electron microscopy. Dissected HPs were fixed in 2.5% glutaraldehyde/2.5% paraformaldehyde in cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated through a graded series of alcohol and embedded in LR White resin. Ultrathin sections were stained with 5% uranyl acetate/50% methanol and Reynold's lead citrate, and viewed on a Hitachi H-800 transmission electron microscope.

RESULTS

MBV-infected HPs

Toluidine blue stained sections showed histopathology typical of MBV, with enlarged nuclei thinly surrounded by cytoplasm or disintegrating cytoplasm (data not shown). Infected nuclei examined in this study contained 1 to 3 OBs. Transmission electron microscopy of infected HPs revealed large numbers of unoccluded virions in enlarged nuclei (Fig. 1a). Virions tended to be distributed in groups throughout the nucleus, rather than close to the nuclear membrane or nucleolus, as observed by Johnson & Lightner (1988). Mature virions were also evident within OBs, but not as densely packed as in the nucleoplasm. At high magnification the OBs revealed the individual polyhedrin subunit structure (Fig. 1b). Subunits were approximately 12 nm in diameter. What appeared to be extensive vacuolation at the periphery of the nuclear membrane was also seen (Fig. 1a).

Ultrastructure of mature virions

Mature virions were rod-shaped, enveloped particles similar to those reported for PmSNPV (Johnson & Lightner 1988, Chen et al. 1989). Envelopes of some virions showed apparent apical cone-shaped projections (Fig. 2a) as previously reported (Johnson & Lightner 1988), reflecting the underlying nucleocapsid form. In addition, many virions had lateral, subapical envelope expansions, some of which appeared to contain a reflexed filament (Fig. 2b). Longitudinal sections of apparent reflexed filaments have previously been reported for crustacean baculoviruses (Johnson 1988,

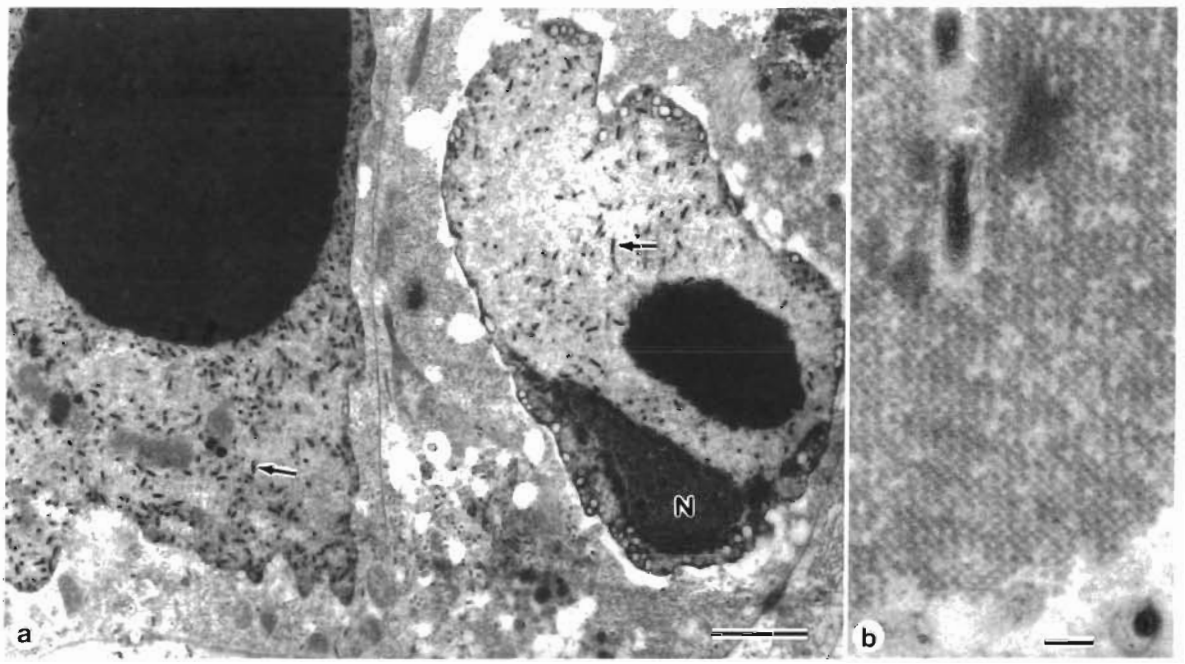

Fig. 1. (a) Monodon baculovirus (MBV) infecting *Penaeus monodon*. Two adjacent MBV-infected hepatopancreas (HP) cells. Note abundant unoccluded virions in nucleoplasm (arrows), and enlarged nucleolus (N). Scale bar = 2 µm. (b) Occlusion body (OB), showing polyhedrin matrix and scattered virions. Scale bar = 100 nm

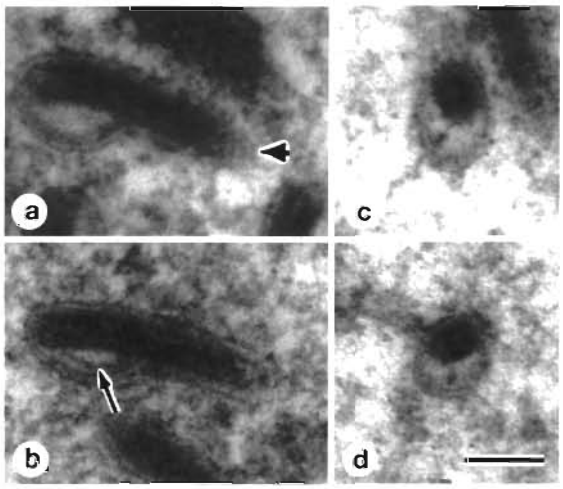

Fig. 2. Ultrastructural features of mature virions. (a) Virion, longitudinal section. Note envelope, electron-dense nucleic acid core, and cone-like extension (arrowhead). (b) Virion showing subapical envelope expansion, containing a filament (arrow). (c, d) Virions in cross-section, showing filament. Scale bar = 100 nm

Johnson & Lightner 1988), but are difficult to distinguish from amorphous matrix protein. In this study, these filaments were occasionally revealed in cross-sections through virions (Fig. 2c, d). Virions were approximately 325 × 70 nm.

Development of virions

Forms which resembled 'capsid originators', described by Johnson (1988) as membranous structures containing a developing capsid, were seen in this study (Fig. 3a). Capsids apparently lacking viral nucleic acid were also observed (Fig 3b). Some contained a central filament, which was seen in both longitudinal (arrowed in Fig. 3b) and cross (Fig. 3c) sections. One of these appeared to extend beyond the normal capsid length (Fig. 3b). A capsid which was apparently in the process of filling with nucleic acid was seen in which only a portion of the internal chamber was electron dense (Fig. 3d). The envelope appeared incomplete at the empty part of the capsid, but was closely apposed at the adjacent electron-dense end. Many virions were seen which appeared to have terminal expansions of the envelope at both ends, imparting a 'dumbell' shape to the particle (Fig. 3e). These occurred in the same EM section as mature virions, and were occasionally found in the same micrograph, suggesting that they are not an artefact.

Naked electron-dense nucleocapsids in the cytoplasm and attached to the nuclear envelope

Unenveloped electron-dense nucleocapsids were found in the cytoplasm of MBV-infected hepatopan-

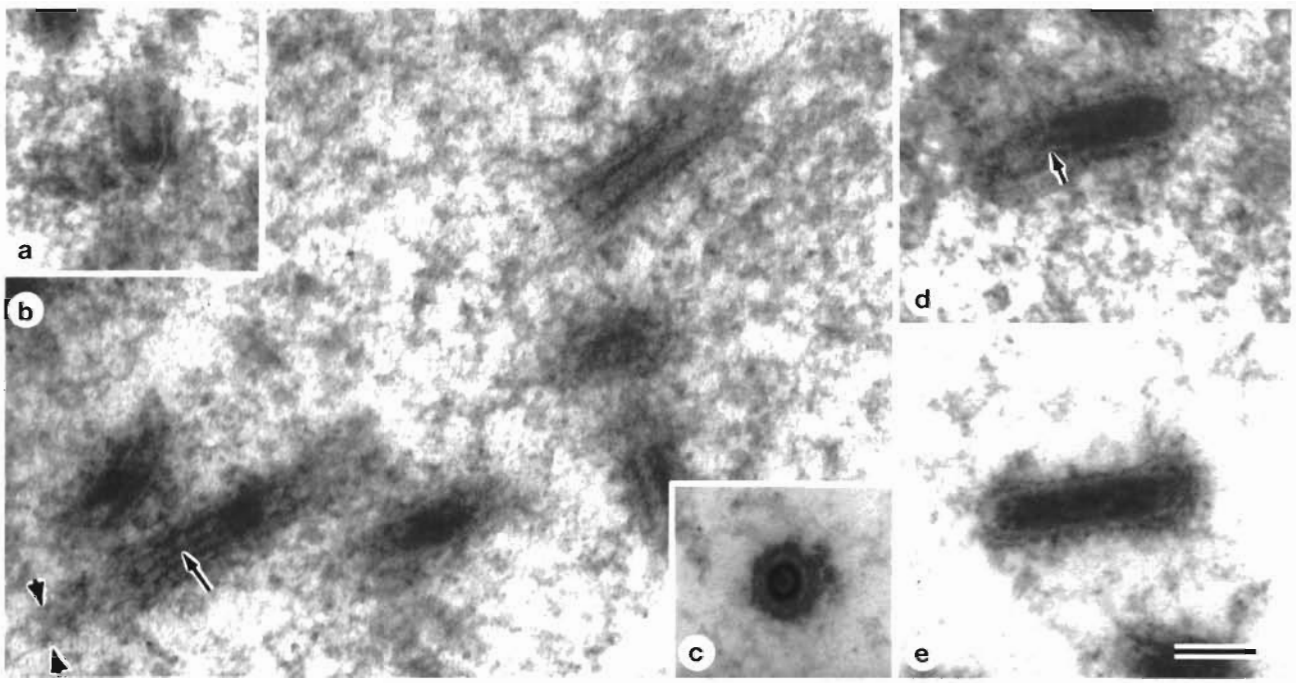

Fig. 3. Putative developmental stages of MBV. (a) Putative capsid originator. (b) Empty capsids. Note central filament (arrow), and longitudinal extension of capsid (arrowheads). (c) Cross-section of capsid. Note filament. (d) Developing virion apparently partly filled with nucleic acid. Note filament (arrow). (e) Dumbell-shaped virion, with envelope detached at both ends. Scale bar = 100 nm

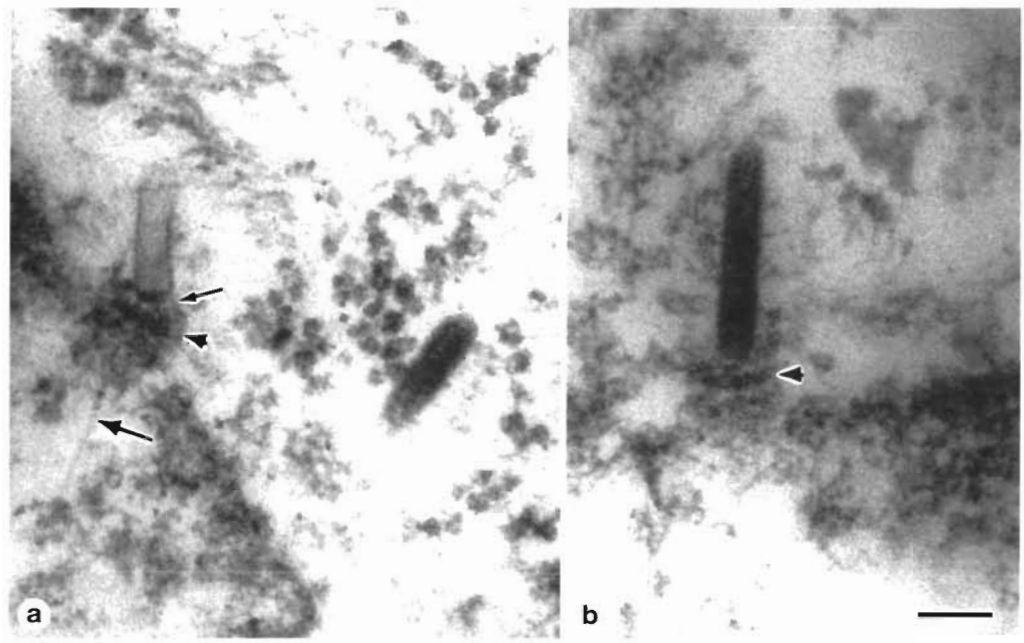

Fig. 4. Nucleocapsids and capsid in cytoplasm. (a) Nucleocapsid in cytoplasm, and empty capsid attached via filaments (small arrow) to electron-dense structure (arrowhead). Note suggestion of thin strand of material (presumably viral DNA or genome) extending into nucleus (large arrow). (b) Nucleocapsid attached to nuclear envelope at electron-dense structure (arrowhead). Scale bar = 100 nm

creatocytes, both free (Figs. 4a & 5b,d) and attached to the nuclear membrane (Fig. 4b). These nucleocapsids appeared to have apical caps. One nucleocapsid was found attached to the nuclear membrane at a platform-like structure composed of diffuse electron-dense material (Fig. 4b), which might have been a remnant of the nuclear pore. This nucleocapsid, which showed 1 conical and 1 blunt end, was 310 nm in length and 45 nm wide and was attached to the nuclear membrane at the blunt end. The particle was linear, as opposed to enveloped nucleocapsids, which often appeared slightly curved (Fig. 2a,b). Its dimensions were similar to those of nucleocapsids within envelopes (Fig. 2a), which were approximately 295 nm in length (maximum 320 nm) and 44 nm in width. The nucleocapsid was slightly more electron dense at the end closer to the nuclear membrane. An empty capsid (44 nm wide) was found attached via filaments to a similar structure, with a strand of material that may be a part of the viral genome extending into the nucleus (Fig. 4a). Similar platform-like structures, found in the same section with no nucleocapsids attached (arrowed, Fig. 5b), were probably sites where the plane of section did not reveal the attached capsid or nucleocapsid, or they had already detached.

In-folding of nuclear membrane

The nuclear membrane of MBV-infected hepatopancreatocytes often appeared locally invaginated (Figs. 1a & 5a). In-folding produced channels and pockets (Fig. 5b,c), and appeared in some cases to pinch off an entire, or almost entire vesicle of cytoplasm (Figs. 1d & 5a). The enclosures contained small particles resembling free ribosomes or polyhedrin subunits (Fig. 5c,d). Microfilaments (Fig. 5b,c) and, in one case, a nucleocapsid (Fig. 5b), were also observed in the invaginations. Similar structures were also seen within the nucleus (Fig. 5c).

Advanced stage of infection

In advanced infections, thin layers of cytoplasm containing abundant free ribosomes surround infected nuclei (Fig. 6). An unusual hepatopancreatocyte was seen in which the nucleoplasm was condensed into areas containing masses of virions (Fig. 6). The nucleoplasm of this cell was similar to that observed by Lightner et al. (1983) within an autophagosome.

DISCUSSION

This report describes ultrastructural observations on MBV of *Penaeus monodon* from Australia. Naked nucleocapsids that are electron dense and thus appear to contain viral genome are shown free in the cytoplasm and attached to the nuclear membrane. These nucleocapsids have not been previously described for MBV, and suggest a means by which the viral genome is transported from the plasma membrane of the hepatopancreatocyte to its nucleus. The presence of these nucleocapsids and naked, empty nucleocapsids at the nuclear membrane suggests that the envelope is

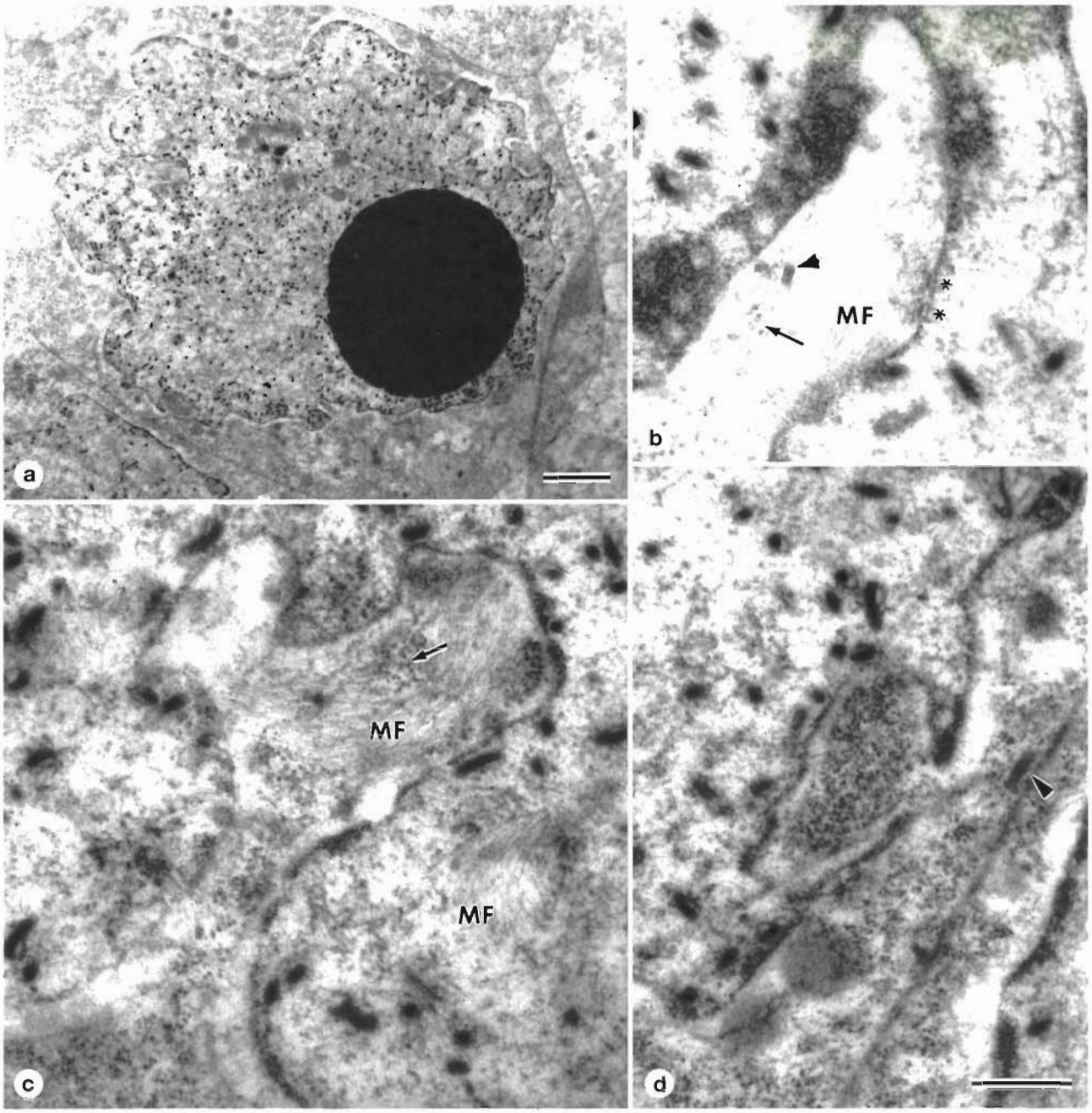

Fig. 5. In-folding of nuclear envelope in MBV-infected HP cells. (a) HP cell in advanced stage of infection showing convolutions of nuclear envelope. (b) Invagination containing microfilaments (MF) and small particles (arrow). Note nucleocapsid (arrowhead) and platform-like structures (*); see text. (c) Doubly infolded channel. Note similar MFs in nucleus and cytoplasm. (d) Pocket of cytoplasm almost entirely surrounded by nuclear membrane. Scale bars: (a) = 2 µm; (b, c, d) = 500 nm

lost at the plasma membrane and the nucleocapsid travels through the cytoplasm and attaches to the nuclear membrane, from where the viral genome enters the nucleus.

Previous studies have provided evidence that this mechanism may operate for MBV and other crustacean baculoviruses. A naked, empty capsid aligned at the nuclear pore was reported by Johnson & Lightner (1988), with a strand of material (presumably nucleic acid) similar to that reported in the present study (Fig. 4a) streaming through the pore. Negatively stained images of unenveloped nucleocapsids of MBV from Thailand show similar filaments (Flegel et al. 1992). Unenveloped nucleocapsids of uneven electron density that are attached to the nuclear pore have been reported for *Baculovirus penaei* (Couch 1991). How-

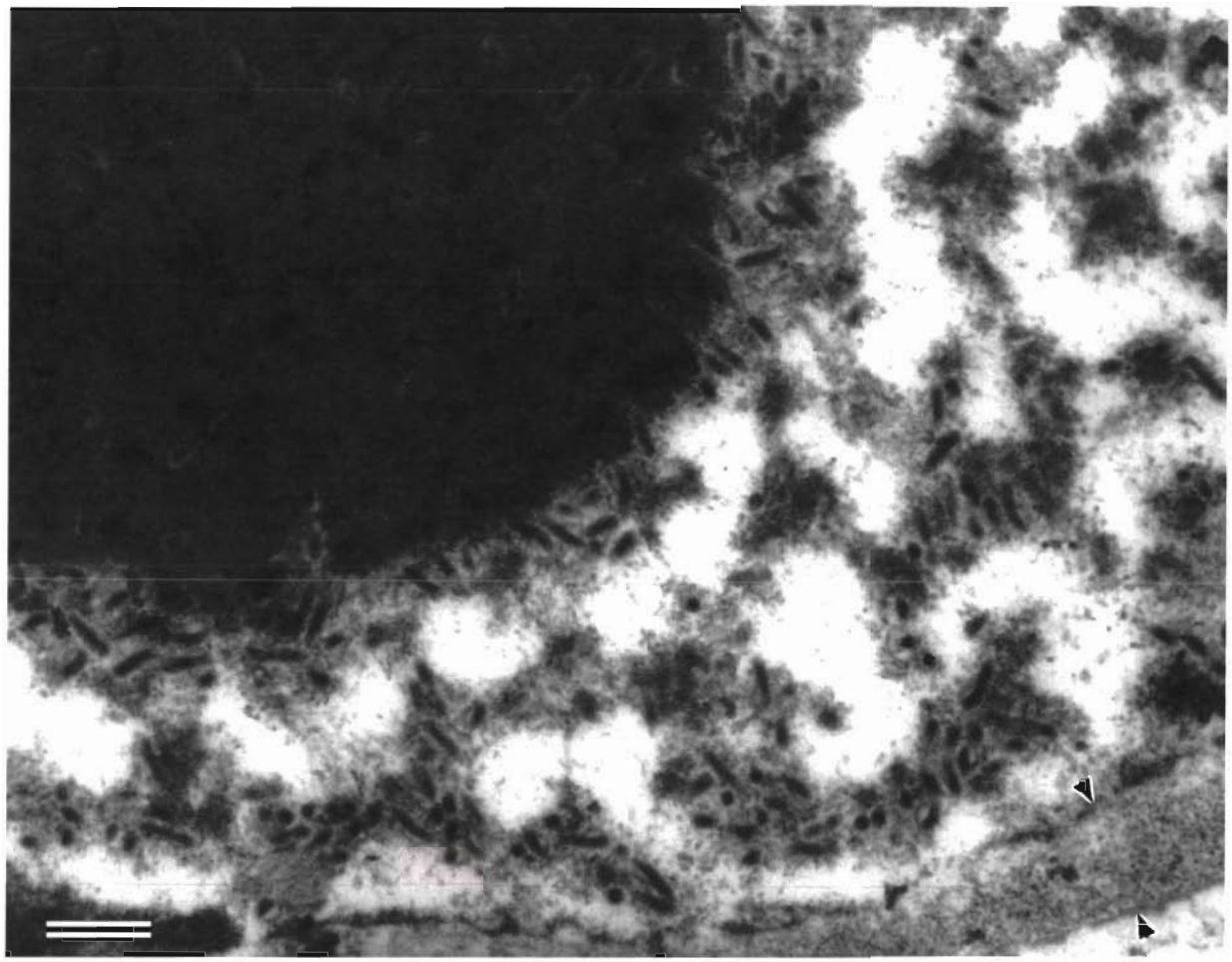

Fig. 6. HP cell in advanced stage of infection. Note condensed nucleoplasm containing virions, large OB and thin shell of dense cytoplasm (arrowheads). Scale bar = 500 nm

ever, invasion of the cell is best documented for the baculovirus of *Carcinus mediterraneus* (Mari 1987) with baculovirus particles shown attached to the cellular membrane, and nucleocapsids to the nuclear membrane. Ultrastructural studies indicate that nuclear polyhedrosis (Adams & McClintock 1991) and granulosis (Tanada & Hess 1991) viruses of insects lose their envelopes at the cellular membrane with unenveloped nucleocapsids traversing the cytoplasm, attaching via a filament at a nuclear pore and releasing the viral nucleic acid into the nucleus. Our observations and those of Johnson & Lightner (1988) suggest that a similar mechanism is likely to operate in MBV.

Features described here, along with those described previously (Doubrovsky et al. 1988), suggest a close relationship of this virus with MBV from other areas of the world (Lightner et al. 1983, Johnson & Lightner 1988, Chen et al. 1989, Vogt 1992). Two transient stages of virion development not shown previously for MBV from Australia are virions partly filled with nucleic acid (Fig. 3d) and dumbell-shaped virions (Fig. 3e). Virions that were partly filled with nucleic acid showed a loose envelope at the unfilled portion, but a closely apposed envelope on the full portion, thus supporting the hypothesis that envelopes may be terminally expanded early in virion assembly, then tighten around the nucleocapsid as the virion matures. Envelopes which expanded around both virion apices (Fig. 3e) were also evident in Baculo-PP, a hepatopancreatic baculovirus of *Paralithoides platypus* (Johnson & Lightner 1988). These forms are not likely to be a fixation artefact, as they were present in the same section, and sometimes in the same photograph as mature virions with closely apposed envelopes.

The partially formed particles present in cells in an advanced stage of infection may be developing virions, or degraded virions, or may result from the overproduction of viral proteins. The nucleocapsids in the cytoplasm and these attached to the nuclear membrane of hepatopancreatocytes that contained OBs are

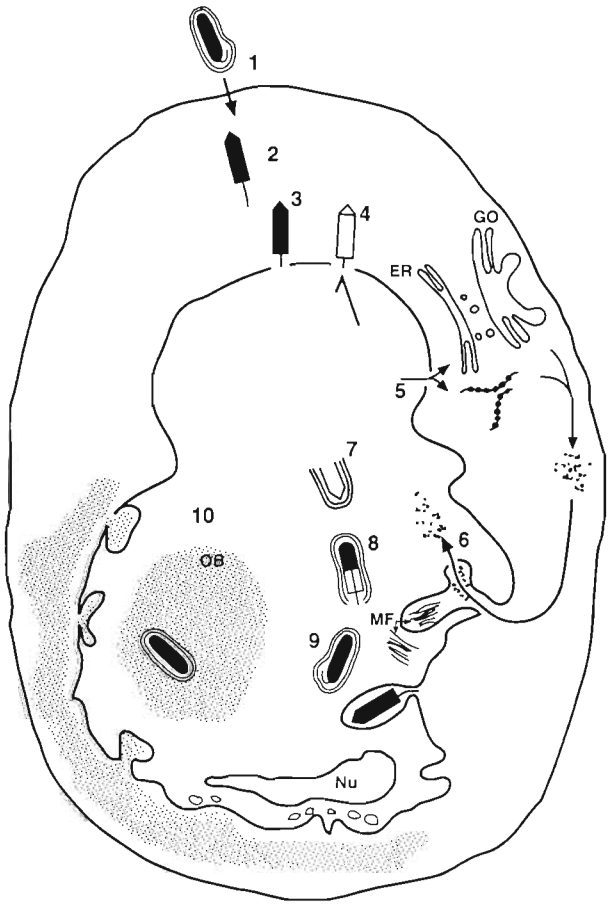

Fig. 7 Proposed model for MBV viral life cycle. (1) Entry of nucleocapsid into cytoplasm and (2) progression to nuclear membrane. (3) Attachment of nucleocapsid to nuclear pore. (4) Transfer of viral genome from capsid to nucleoplasm. (5) Transport of viral mRNA from nucleoplasm to cytoplasm, followed by synthesis of viral proteins by ribosomes either free or on the endoplasmic reticulum (ER) with further processing through the Golgi apparatus (GO). (6) Transport of viral proteins and polyhedrin into nucleus. (7) Formation of capsid originator, with further assembly into capsid. (8) Entry of viral genome into capsid. (9) Tightening of envelope around virion apex and formation of subapical envelope pouch containing a filament. (10) Occlusion of some of the virions in developing OB. Possible entry of additional viral nucleic acid into already infected cell is shown at invaginated pocket. Nu = nucleolus

consistent with the on-going viral infection of cells that are already heavily infected.

Capsids extending to twice the normal length (Fig. 3b) have not been previously seen in electron micrographs of thin-sectioned MBV, but are reported in negatively stained material for *Baculovirus penaei* (Bonami et al. 1995). Abnormally long capsids have also been reported for rod-shaped virus of *Carcinus maenus* (RV-CM) (Johnson 1988).

Johnson & Lightner (1988) and Doubrovsky et al. (1988) commented on the irregular appearance of the nuclear envelope in advanced MBV infections, and similar convolutions can be seen in low magnification micrographs in other publications (Chen et al. 1989, Vogt 1992). In-folding of the nuclear envelope might possibly play a role in the transport of virion-associated proteins from the cytoplasm into the nucleus. However, since these were most pronounced in advanced infections, they may represent the passive folding of the enlarged, virus-filled nucleus about the remnants of the cytoskeletal framework of transformed HP cells.

Following protein transport into the nucleus, microtubules and microfilaments (Fig. 5b,c), similar to those which occur in the cytoplasm of baculovirus-infected HP cells (MBV, Lightner et al. 1983) and haemocytic RV-CM (Johnson 1988), are apparently assembled. Their function in the nucleus is not known. Microfilaments were almost always present in pockets formed by deep invaginations, suggesting the possibility of a role for microfilaments in induction of infolding.

Observations in this publication and others support the model for virogenesis and replication depicted in Fig. 7. Insect baculoviruses enter the cytoplasm of host cells by plasma membrane fusion and loss of envelopes, or absorptive endocytosis (Adams & McClintock 1991), but the mechanism is as yet unknown for MBV (1). Naked nucleocapsids (Figs. 4a & 5b,d) traverse the cytoplasm (2), attach to the nuclear membrane (3) possibly at the nuclear pore (Fig. 4a; Johnson & Lightner 1988) and transfer their nucleic acid into the nucleus (4). Transcription of the viral genome leads to mRNA coding for viral proteins. This mRNA exits the nucleus via nuclear pores, and viral proteins are synthesised in the cytoplasm (5) and transported into the nucleus (6) via nuclear targeting sequences (Lewin 1994). Infolding of the nuclear membrane may play some contributory role here. Capsids begin to develop (Johnson & Lightner 1988; Fig. 3a) in conjunction with envelopes (7). A central filament forms (Fig. 3b), the viral genome fills the capsid (8; Fig. 3d) and the expanded apical envelope at the anterior end (Fig. 3e) becomes closely apposed to the virion core. The extended apical envelope at the posterior end develops into the lateral envelope expansion (9), which holds the terminal filament (Fig. 2b). Some of the virions become enclosed in the developing OB (10). Additional nucleocapsids may discharge their nucleic acid into already infected cells. Finally, the nuclear envelope disintegrates (Doubrovsky et al. 1988, Lightner et al. 1983), releasing virions and OBs. This stage may in some cases be preceded by a condensation of nucleoplasm into virion-containing patches (Fig. 6).

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