# Advances in the Molecular Biology of Baculoviruses

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## Abstract

*Baculoviridae* constitutes a family of insect-specific, large DNA viruses with a unique life cycle characterized by the production of two morphologically distinct virions, the budded virus (BV) and the occlusion-derived virus (ODV). ODV and BV, with different tissue tropisms, have been widely applied in the areas of biological control and biotechnology, respectively. In nature, baculovirus infection of susceptible host larvae is initiated by ODV-mediated primary infection, followed by the production of BV for spreading infection within larval body. Across millions of years of co-evolution with their hosts, baculoviruses have developed dedicated mechanisms for efficient entry/egress, genome replication/transcription, and virion assembly by employing either their own proteins or host machineries. They have also adopted versatile strategies to precisely regulate the immunity, behaviours and physiology of hosts to facilitate their own replication and dispersal. In this chapter, research advances relating to key aspects of the baculovirus life cycle are reviewed, and the application of a newly-developed baculovirus synthetic biology technology is introduced. Finally, future avenues for baculovirus research are discussed.

## Introduction

*Baculoviridae* constitutes a family of insect-specific large DNA viruses with rod-shaped, enveloped virions. The family is divided into four genera, *Alphabaculovirus* [lepidopteran-specific nucleopolyhedroviruses (NPVs)], *Betabaculovirus* [lepidopteran granuloviruses (GVs)], *Gammabaculovirus* (hymenopteran NPVs), and *Deltabaculovirus* (dipteran NPVs).

A typical baculovirus life cycle produces two morphologically distinct virion phenotypes: the budded virus (BV) and the occlusion-derived virus (ODV). The ODV is embedded within occlusion bodies (OBs) and is specifically responsible for mediating infection of midgut columnar epithelial cells (known as 'primary infection', 'oral infection' or '*per os* infection'). The BV is produced from midgut epithelial cells and infects other tissues within the larval body (termed as 'secondary infection' or 'systemic infection') (Slack and Arif, 2007). BVs and ODVs share identical genomes but differ with regard to their protein composition, especially the envelope proteins, which may determine the different tissue-tropisms of these two virion phenotypes (Hou *et al.*, 2013). A comparative model presenting protein compositions and localizations of BV and ODV based on experimental data for *Helicoverpa armigera nucleopolyhedrovirus* (HearNPV) is shown in Fig. 9.1. OBs containing ODVs have been widely used as bio-insecticides to control insect pests, whereas BVs have important applications as vectors for protein expression and gene delivery. A successful baculovirus infection depends on delicate interactions between virus and host at different stages of infection including

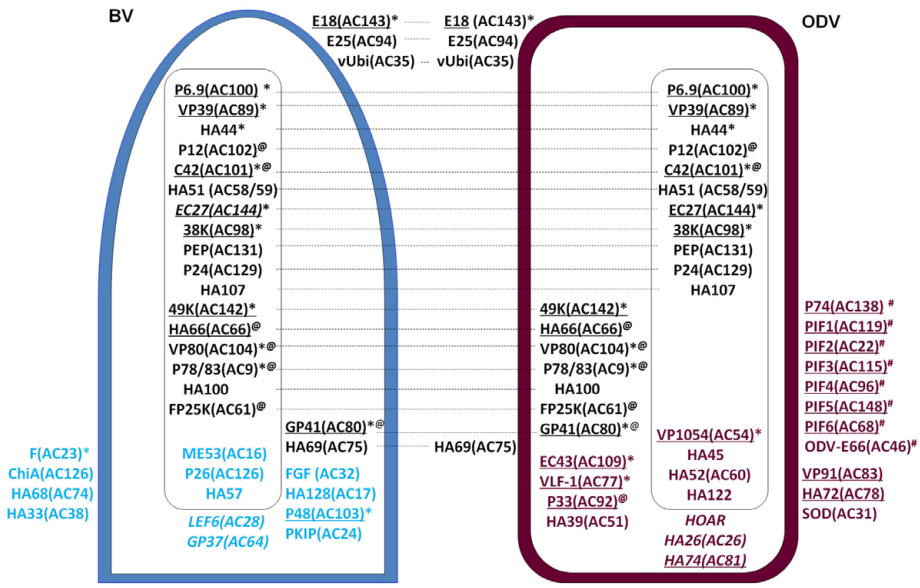


**Figure 9.1** Comparative schematics showing protein composition and localization of HearNPV BV and ODV based on proteomics data. Envelope proteins are shown on the surfaces of BV and ODV, nucleocapsid proteins are shown inside each rectangle, and EC proteins are located in the space between the envelope and the nucleocapsid. Proteins shared by BV and ODV are in black; those proteins located in both BV and ODV are linked by dashed lines; BV-specific proteins are shown in blue; and the ODV-specific proteins are shown in maroon. The underlined proteins are conserved baculovirus core genes, * denotes proteins essential for BV production, @ denotes proteins involved in nucleocapsid and/or protein traffic, and # denotes proteins involved in oral infection. BV and ODV proteins with unknown locations are shown in italics. The ORF numbers of the AcMNPV homologues for all the HearNPV ORFs are indicated in brackets. (Reprinted from Hou *et al.*, 2013, J Virol. *87*, 829–839.)

entry, intracellular transport, genome replication/transcription, virion assembly and egress. In addition, baculoviruses have developed strategies to regulate the immunity, behaviours and physiology of hosts to facilitate the production of virus progeny and dispersal. Research advances in these areas are summarized below.

## Baculovirus genome

To date, baculoviruses from more than 700 host species have been described. Among these, more than 172 sequenced complete baculovirus genomes have been reported, including 127 alphabaculoviruses, 41 betabaculoviruses, 3 gammabaculoviruses, and 1 deltabaculovirus (Wennmann *et al.*, 2018). The baculovirus genome consists of a double-stranded (ds), covalently closed, circular DNA molecule ranging from 80–180 kbp, with 90–180 predicted open reading frames (ORFs) tightly organized in either orientation. Among these, *Xestia c-nigrum granulovirus* (XecnGV) has the largest genome, with approximately 179 kbp encoding 181 ORFs; gammabaculovirus genomes are the smallest, with approximately 80 kbp and around 90 predicted ORFs. A total of 38 conserved core genes are present in all the baculoviruses sequenced to date (Javed *et al.*, 2017). Most baculovirus genomes also contain short homologous repeats (*hrs*). Fig. 9.2 shows the genome organization of the baculovirus type species, *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV). Notably, a collinear region containing approximately 20 core genes is conserved across all lepidopteran baculoviruses (Zhu *et al.*, 2018) (Fig. 9.2).

## Baculovirus core genes

Although different baculovirus genomes exhibit marked diversity with regard to gene content and order, the 38 core genes present in all sequenced baculovirus genomes can be classified into four categories: DNA replication, transcription, oral

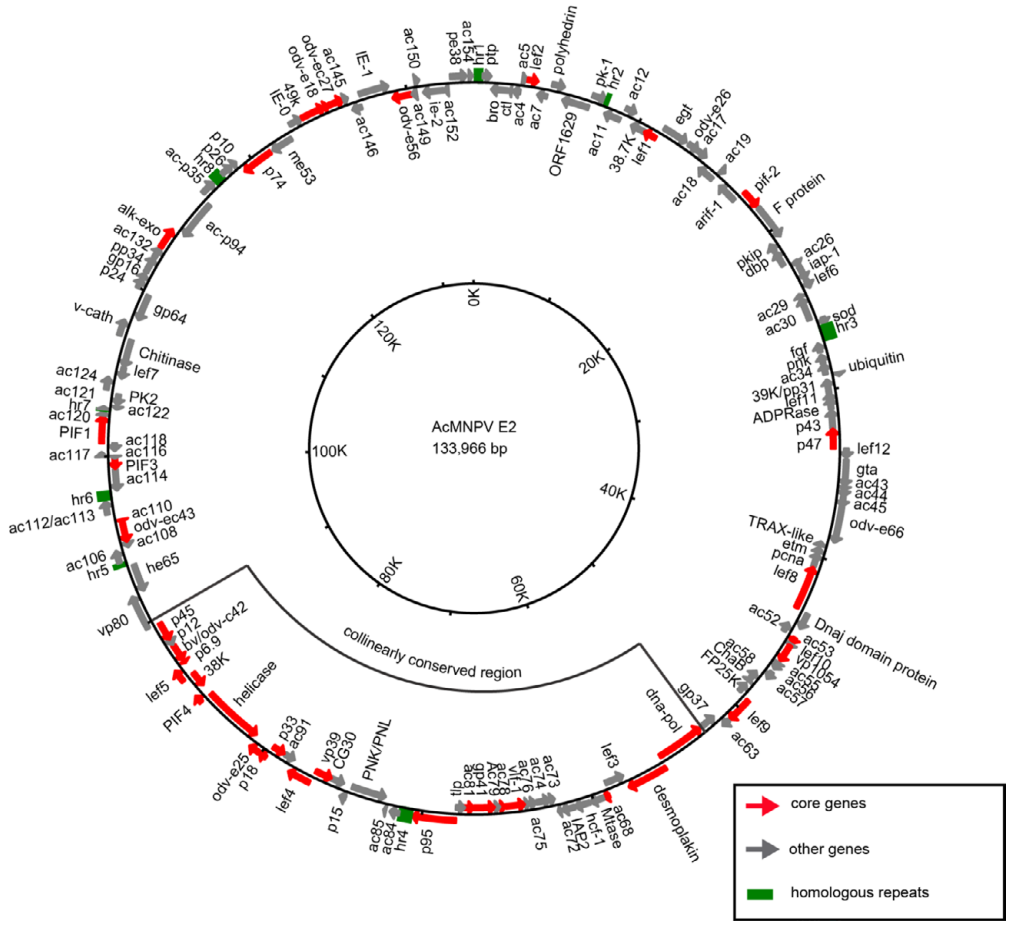


**Figure 9.2** Circular map of AcMNPV genome. The complete circular dsDNA genome of AcMNPV E2 strain is 133,966 bp (GenBank accession number: KM667940) with a G + C content of 40.7%. The genome contains 154 ORFs including 38 core genes (red), and 8 *hrs* (green). The lepidopteran linearly conserved region is indicated.

infection, and structure/assembly (Table 9.1). Among the core genes, five are related to DNA replication, including *lef1*, *lef2*, DNA polymerase (*dnapol*), helicase (*p143*) and alkaline nuclease (*an*). Six core genes are involved in transcription, including the four subunits of viral RNA polymerase. Nine core genes are *per os* infectivity factors (*pifs*), including *p74* (*pif0*), *pif1–7* and *pif8* (*vp91*). The remaining 18 core genes are related to nucleocapsid assembly, BV formation or ODV morphogenesis. Notably, some of the core genes are also found in other invertebrate DNA viruses, including *White spot syndrome virus* (WSSV), ascovirus, nudivirus, and hytrosavirus, suggesting a common origin of these large DNA viruses and the occurrence of gene transfer between different viruses and their insect hosts.

## Homologous repeats

*Hrs* are characterized by AT-rich and direct repeats along with imperfect palindromes that are interspersed in baculovirus genomes (Kool *et al.*, 1993; Hilton and Winstanley, 2008). The sequences and numbers of *hrs* vary considerably among different baculoviruses. Certain baculoviruses, e.g. *Trichoplusia ni single nucleopolyhedrovirus* (TnSNPV) and *Agrotis segetum granulovirus* (AgseGV), do not appear to contain *hrs*, whereas others contain many, such as *Cydia pomonella granulovirus* (CpGV), which has 13. *Hrs* function as transcriptional enhancers for early gene expression and as origins of DNA replication (Guarino and Summers, 1986; Pearson *et al.*, 1992, 1993). The transcriptional activator IE-1 can bind to *hrs* and enhance the activity of *hr*-adjacent viral promoters (Olson *et al.*, 2001, 2003). Although

**Table 9.1** The 38 core genes of baculovirus

| Catalogue (Total no.) | Gene (AcMNPV homolog) | Protein function |
|---|---|---|
| DNA replication (5) | *lef2* (*ac6*) | A primase-associated factor |
| | *lef1* (*ac14*) | primase |
| | *dnapol* (*ac65*) | DNA polymerase |
| | *helicase* (*ac95*) | Helicase |
| | *alkaline nucleus* (*ac133*) | DNA recombination |
| Transcription (6) | *p47* (*ac40*) | RNA polymerase subunit |
| | *lef8* (*ac50*) | RNA polymerase subunit |
| | *lef9* (*ac62*) | RNA polymerase subunit |
| | *vlf-1* (*ac77*) | Very late gene expression |
| | *lef4* (*ac90*) | RNA polymerase subunit |
| | *lef5* (*ac99*) | Late gene transcription |
| Oral infection (9) | *p74/pif0* (*ac138*) | PIF complex, ODV binding? |
| | *pif1* (*ac119*) | PIF complex, ODV binding? |
| | *pif2* (*ac22*) | PIF complex, ODV binding? |
| | *pif3* (*ac115*) | PIF complex, unknown function |
| | *pif4* (*ac96*) | PIF complex, unknown function |
| | *pif5* (*ac148*) | unknown function |
| | *pif6* (*ac68*) | PIF complex, unknown function |
| | *pif7* (*ac110*) | PIF complex, unknown function |
| | *vp91/pif8* (*ac83*)* | PIF complex, unknown function |
| Structure-related (18) | *ac53* | Nucleocapsid assembly |
| | *vp1054* (*ac54*) | Nucleocapsid assembly |
| | *desmoplakin* (*ac66*) | Nucleocapsid egress |
| | *ac78* | BV production and ODV morphogenesis |
| | *gp41* (*ac80*) | Tegument protein, BV production and ODV morphogenesis |
| | *ac81* | BV production and ODV envelopment |
| | *vp39* (*ac89*) | Major capsid protein, nucleocapsid assembly |
| | *p33* (*ac92*) | sulfhydryl oxidase, BV production and ODV morphogenesis |
| | *p18* (*ac93*) | BV production and ODV envelopment |
| | *odv-e25* (*ac94*) | BV infectivity and ODV formation |
| | *38k* (*ac98*) | Phosphatase, nucleocapsid assembly |
| | *p6.9* (*ac100*) | DNA condensation |
| | *bv/odv-c42* (*ac101*) | Nucleocapsid assembly and actin polymerization |
| | *p48/p45* (*ac103*) | BV production and ODV envelopment |
| | *odv-ec43* (*ac109*) | BV production and ODV envelopment |
| | *49k* (*ac142*) | BV production and ODV envelopment |
| | *odv-e18* (*ac143*) | BV production |
| | *odv-ec27* (*ac144*) | Nucleocapsid assembly and cell cycle arrest |

**vp91* is also involved in nucleocapsid assembly.

deletion of each of the 8 *hrs* or a double *hr* deletion (*hr2* and *hr3*) in AcMNPV did not impair BV infectivity (Carstens and Wu, 2007), simultaneous elimination of all eight *hrs* of AcMNPV using a sequential deletion strategy resulted in a 100-fold reduction of infectious BV production, as well as

severe impairment of OB production (Bossert and Carstens, 2018). It was therefore proposed that three *hrs* may be sufficient to mediate optimal virus infection (Bossert and Carstens, 2018). *Hrs* are also present in the genomes of other invertebrate DNA viruses (e.g. nimavirus, ascovirus, nudivirus, and iridovirus), suggesting a common function of *hrs* in these invertebrate large dsDNA viruses.

## PIF-mediated ODV entry into midgut cells (primary infection)

In nature, ODVs initiate primary infection when they are released from ingested OBs in the alkaline conditions within insect midguts. Unlike BVs, which use only one envelope fusion protein (EFP) (GP64 or F protein) for infection, ODVs employ a group of specific envelope proteins called *per os* infectivity factors (PIFs) to mediate primary infection. Deletion of any one of the individual PIFs leads to the complete loss of oral infectivity. PIFs form a stable protein complex on the surface of ODV. Interestingly, certain PIFs are also present in other invertebrate large DNA viruses, suggesting a common origin and conserved functions of these PIFs. The current knowledge of the *per os* infection process and PIFs has been summarized in detail in recent reviews (Boogaard *et al.*, 2018; Wang, X. *et al.*, 2017; Wei *et al.*, 2014).

### PIFs

Nine reported PIFs (P74 or PIF0, and PIF1–8) are conserved in all baculoviruses sequenced to date (Table 9.1). They were named in the order in which they were identified as being essential for oral infectivity. These PIFs do not show obvious sequence similarity and vary considerably in their molecular mass (ranging from ≈ 7 kDa to ≈ 95 kDa) (Wang, X. *et al.*, 2017). However, they do share some common features. All PIFs contain one or more hydrophobic transmembrane (TM) domains. Some of these TMs serve as an inner nuclear membrane sorting motif (INM-SM) and are responsible for the trafficking of PIFs from the cytoplasm to the inner nuclear membrane and ODV envelope (Braunagel and Summers, 2007). Additionally, most PIFs contain conserved cysteines, which may be involved in disulfide bond formation, thus stabilizing the interaction between PIFs or facilitating the proper folding of individual PIFs (Wang, X. *et al.*, 2017).

Direct membrane fusion between the ODV envelope and the midgut cell plasma membrane is likely involved in ODV entry into cells. However, the precise role of each PIF during *per os* infection remains largely enigmatic. Among PIFs, P74, PIF1, and PIF2 have been suggested to play a role in the specific binding of ODV to midgut cells (Ohkawa *et al.*, 2005). However, another study by using EGFP-labelled ODVs showed that the deletion of either *pif1* or *pif2* did not prevent virus binding to midgut cells (Mu *et al.*, 2014). P74 shares a common feature with class I viral fusion proteins in that it undergoes proteolysis for activation (Slack *et al.*, 2008; Peng *et al.*, 2011). However, whether P74 serves as an actual fusion protein for ODV entry is unclear.

### PIF complex

PIFs are likely to orchestrate the entry of ODVs into midgut epithelial cells by formation of a complex, a similar mechanism to that utilized by other large DNA viruses such as poxviruses and herpesviruses. Peng *et al.* (2010a) provided the first evidence for the presence of a stable PIF complex (> 170 kDa) under partially denaturing conditions. In 2012, the same group determined that the PIF complex is as large as ≈ 480 kDa under non-denaturing conditions (Peng *et al.*, 2012). The components of the PIF complex have been discovered successively, including 8 PIFs (PIFs 0 to 4 and PIFs 6 to 8), i.e. all the PIFs except PIF5 (Peng *et al.*, 2010a, 2012; Javed *et al.*, 2017; Boogaard *et al.*, 2018). Among these, PIFs 1–4 are proposed to form a stable core (Boogaard *et al.*, 2018). PIFs interact extensively with each other, as well as with themselves (Peng *et al.*, 2010b; Dong *et al.*, 2014; Zheng *et al.*, 2017). HearNPV PIFs 0 through 3 could not be functionally substituted with homologues from other baculoviruses, suggesting that the interactions between PIFs are highly specific (Song *et al.*, 2016; Makalliwa *et al.*, 2018). It has been suggested that the formation of an intact PIF complex may prevent degradation of individual PIF proteins in the proteolytic environment of the insect midgut lumen (Boogaard *et al.*, 2017). Many details of the formation and structure of the PIF complex remain unknown.

### Conservation of PIFs

Interestingly, homologues of certain PIFs are present in other invertebrate large DNA viruses, including WSSV (P74 and PIF1–3), salivary gland

hypertrophy viruses (P74, PIF1–4), Apis mellifera filamentous virus (P74, PIF1–4 and PIF8), bracovirus and nudivirus (P74, PIF1–6 and PIF8) (Wang, X. *et al*., 2017). These viruses have co-evolved with their invertebrate hosts over 300 million years ago, implying that PIF-mediated virus entry is an ancient and evolutionarily conserved mechanism shared by these invertebrate DNA viruses. Whether a similar PIF-complex also exists in other invertebrate large DNA viruses and whether such PIFs also play pivotal roles during virus infection would be of great interest.

## Envelope fusion protein (EFP)-mediated BV entry (systemic infection)

Secondary infection of baculoviruses from midgut ephithelia into other tissues within the susceptible larval body is mediated by the BV phenotype. BVs acquire envelopes containing specific viral EFPs during nucleocapsid budding from the host plasma membrane. EFPs have been found in all baculoviruses, except for members of the *Gammabaculovirus* genus. Baculoviruses encode two types of EFPs: GP64 and F. These two proteins are quite distinct in their distribution, structure, and mode of activation, although they perform similar essential roles in mediating BV entry.

### GP64

GP64 homologues only occur in Group I alphabaculoviruses and a betabaculovirus and they are closely related in amino acid (aa) sequence (> 74% aa identity). The GP64 protein was first identified via a neutralizing monoclonal antibody (AcV1) that could block BV entry of AcMNPV (Hohmann and Faulkner, 1983). GP64 is synthesized as an N-glycosylated protein with molecular mass of approximately 64 kDa and is sufficient to mediate low-pH-dependent membrane fusion (Blissard and Wenz, 1992; Jarvis and Garcia, 1994). The crystal structure revealed that the post-fusion conformation of AcMNPV GP64 is comprised of a trimer with a central helix-core and elongated β-sheets, which resembles the features of class III viral fusion proteins such as herpesvirus gB protein and vesicular stomatitis virus G protein (Kadlec *et al*., 2008). The N-terminal region (21 to 159 aa) of GP64 is implicated as a putative receptor binding domain (RBD) based on neutralization and truncation analyses (Zhou and Blissard, 2008). The C-terminal-most regions of AcMNPV GP64 include a 23-aa pre-transmembrane (PTM) domain, a 23-aa transmembrane (TM), and a very short (7 aa) cytoplasmic tail domain (CTD). Functional analyses show that the PTM and TM domains are crucial for the membrane fusion activity of GP64 and BV infectivity. The 7-aa CTD is not essential for infectious BV production but plays a role in virus budding (Oomens and Blissard, 1999; Li and Blissard, 2008, 2009a,b). Two fusion loops (Y75–T86 and N149–H156) located at the tip of the β-sheet domains of the GP64 trimer are critical for GP64-induced membrane fusion events (hemifusion and fusion pore expansion) (Kadlec *et al*., 2008; Dong and Blissard, 2012). Notably, the fusion loops overlap with the RBD at least at some key residues and certain mutations abolish GP64–liposome interaction at neutral pH. This finding suggests dual functions of the GP64 fusion loops in both receptor binding and membrane fusion (Dong and Blissard, 2012).

GP64 also undergoes various post-translational modifications. Four N-glycosylation sites (N198, 355, 385, and 426) are distributed in AcMNPV GP64, some of which are involved in infectious BV production (Jarvis and Garcia, 1994; Jarvis *et al*., 1998). A palmitoylation site (C503) is located in the TM of AcMNPV GP64, although it does not appear to be involved in BV entry or budding (Zhang *et al*., 2003). Of the 15 total conserved cysteines of GP64, 14 are involved in disulfide bond formation. Among these, the trimeric structure is stabilized by an intermolecular disulfide bond (C24–C372) that is not essential for membrane fusion but is required for BV infectivity. The six conserved intramolecular disulfide bonds are all indispensable for GP64 fusogenicity (Li and Blissard, 2010). Some of these key domains/residues of GP64 are highlighted in the post-fusion structure of AcMNPV GP64 (PDB No. 3DUZ) (Fig. 9.3A).

### F protein

Unlike GP64 Group I alphabaculoviruses, which use GP64 as an EFP, Group II alpha-, beta-, and delta-baculoviruses use F proteins for entry. Compared to those of GP64, F protein homologues are much less conserved (20–40% aa identity). Similar to GP64, the F protein homologues are

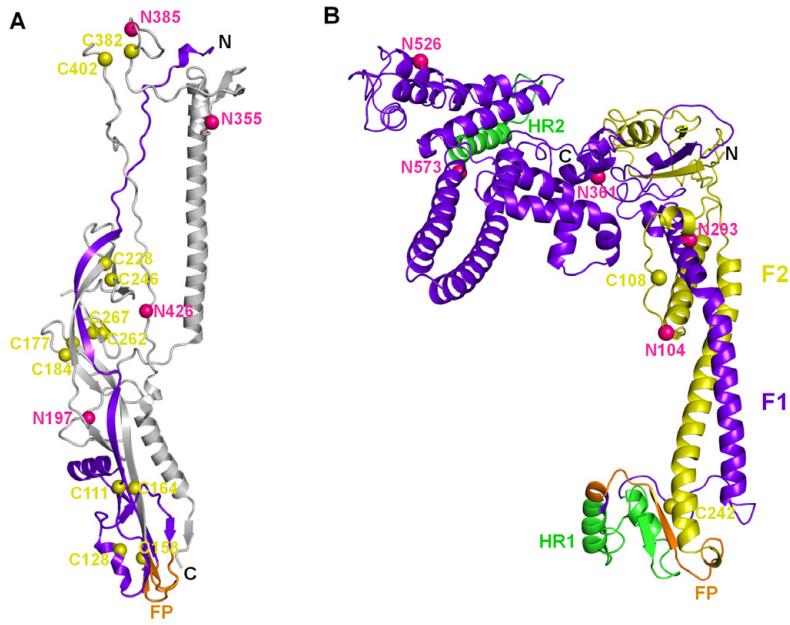


**Figure 9.3** The key structural features of GP64 and F protein. (A) Ribbon diagram of the post-fusion structure of AcMNPV GP64 monomer (PDB entry: 3DUZ). Two fusion loops (residues 75–86 and residues 149–156) and the receptor binding domain (residues 21–159) are coloured orange and purple, respectively. The cysteines forming six intramolecular disulfide bonds and four N-glycosylation sites are shown as spheres and coloured in yellow and crimson respectively. (B) The structure of HearNPV F protein predicted by online I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The cysteines (C108, C242) forming inter-subunit disulfide bonds and five N-glycosylation sites are shown as spheres. Colouring scheme: $F_2$ (residues 1–173) in yellow, $F_1$ (residues 174–677) in purple, fusion loop (residues 174–192) in orange, heptad repeat 1 (HR1) (residues 194–236) and heptad repeat 2 (HR2) (residues 541–568) in blue, cysteines in yellow, N-glycosylation sites in carmine.

N-glycosylated proteins that accumulate at the plasma membranes and possess low-pH induced membrane fusion activity (Pearson *et al.*, 2000; IJkel *et al.*, 2000). The F proteins are expressed as a precursor ($F_0$, approximately 80 kDa), which then undergo proteolysis by host furin-like proprotein convertase to generate a disulfide-linked N-terminus ($F_2$, approximately 20 kDa) and C-terminus ($F_1$, approximately 60 kDa) that are required for activation of F fusogenicity and BV infectivity (Westenberg *et al.*, 2002). A fusion peptide (FP) domain (approximately 20 aa) directly downstream of the furin cleavage site is essential for insertion of the F protein into the cellular membrane, thus facilitating the subsequent fusion between viral envelope and cell membrane (Westenberg *et al.*, 2004; Tan *et al.*, 2008).

Nuclear magnetic resonance (NMR) analysis determined that the FP of HearNPV F (HaF) protein is an amphiphilic structure composed of a 'helix–turn–helix' motif (Tan *et al.*, 2008). At least two heptad repeats (HRs), one located downstream of the fusion peptide and the other located upstream of the transmembrane domain, are present in F proteins, which are presumed to form a six-helix bundle structure for mediating membrane fusion (Long *et al.*, 2008). An inter-subunit disulfide bond (C108–C242) and five N-glycosylation sites (one in the $F_2$ subunit and four in the $F_1$ subunit) were identified in HaF by mutagenesis analysis as being essential for proper function of the F protein and BV infectivity (Yin *et al.*, 2014; Shen *et al.*, 2016). These features suggest the baculovirus F protein is a class I viral fusion protein. However, the 3D structure of F protein, which is important for further in-depth elucidation of its function and fusion mechanism, has yet to be resolved. A post-fusion conformation of the HaF protein has been predicted using the I-TASSER method (Shen *et al.*, 2016) (Fig. 9.3B).

### F-like protein and the evolutionary hypothesis

Notably, Group I alphabaculoviruses also encode an F protein homologue in their genome: F-like protein. F-like proteins share 20–30% aa identity with the F proteins of Group II alpha- and betabaculoviruses. Unlike the functional F protein and GP64, F-like proteins are present in the envelope of both BV and ODV phenotypes (Pearson *et al.*, 2001). Strikingly, F-like proteins accumulate mutations in their furin cleavage sites and are thus unable to induce membrane fusion. These remnant F-like proteins appear to have an auxiliary function in ODV morphogenesis and viral infectivity both *in vivo* and *in vitro* (Lung *et al.*, 2003; Wang, M. *et al.*, 2008; Yu *et al.*, 2009).

F proteins are hypothesized to be the ancestral baculovirus fusion protein and GP64 is likely to have been more recently acquired by Group I alphabaculoviruses. This may have led to the inactivation of F proteins and change into non-fusogenic F-like proteins (Jiang *et al.*, 2009). The first experimental data for this hypothesis was provided by construction of a series of recombinant viruses to mimic the important evolutionary event of acquisition of *gp64* by an ancestral baculovirus (Wang *et al.*, 2014). This hypothesis is further supported by the findings that an ancient deltabaculovirus from mosquito encodes a functional F protein and a newly discovered betabaculovirus possesses not only F protein, but also a functional GP64 homologue (Ardisson-Araujo *et al.*, 2016; Wang, M. *et al.*, 2017). The F protein appears to harbour additional functions over those of GP64 in mediating Group II alphabaculovirus infection, as evidenced by the ability of F proteins to readily replace the function of GP64, but not vice versa (Lung *et al.*, 2002; Long *et al.*, 2006; Westenberg and Vlak, 2008; Wang *et al.*, 2010b). In particular, GP64 was suggested to have been captured from *Thogotovirus*, an arbovirus that also utilizes a GP64 homologue (GP75) as EFP (Pearson *et al.*, 2002). In contrast, F proteins may be derived from their hosts or insect retroviruses, although their functions have since been substantially adapted to baculovirus infection (Lung and Blissard, 2005; Pearson and Rohrmann, 2006).

### Role of EFPs in BV entry

BV entry is initiated by the specific binding of EFPs to susceptible cells (Hefferon *et al.*, 1999). In the case of GP64, abundant cell surface molecules, including phospholipids and heparin, are involved in AcMNPV entry into insect or mammalian cells, respectively (Duisit *et al.*, 1999; Tani *et al.*, 2001; Wu and Wang, 2012). In addition, the host cellular membrane protein BmREEPa facilitates *Bombyx mori nucleopolyhedrovirus* (BmNPV) entry into insect cells through interaction with GP64 (Dong, X.L. *et al.*, 2015). Although it is recognized that F proteins may exploit different cellular receptors from those of GP64 to infect insect cells (Westenberg *et al.*, 2007), the specific receptors for both F protein and GP64 have yet to be identified.

During AcMNPV infection, at 10–20 minutes post-binding, BV particles are internalized into cells by clathrin-mediated endocytosis. Viral nucleocapsids are released from endosomes into the cytoplasm at 15–30 minutes post-infection (min p.i.) (Volkman and Goldsmith, 1985; Hefferon *et al.*, 1999). The low-pH condition inside endosomes is critical for triggering the conformational change of EFPs, which results in fusion between the viral envelope and endosomal membranes. The protonation of histidine residues (H245, 304, and 430) may comprise an important trigger for the low-pH-induced conformational change of GP64 (Li and Blissard, 2011); in turn, five to eight GP64 trimers may act in concert to mediate the initial fusion pore formation (Markovic *et al.*, 1998). The optimal fusion pH for GP64 is around 5.5, implying that virus fusion may occur in late endosomes (Blissard and Wenz, 1992). Furthermore, low-pH-triggered direct fusion with the plasma membrane appears to serve as an alternative pathway for AcMNPV entry into insect and mammalian cells (Dong *et al.*, 2010).

## Essential functions of actin polymerization in baculovirus infection

Baculovirus infection induces the sequential rearrangement of host actin in both the cytoplasm and nucleus (Charlton and Volkman, 1991). Unlike most other viruses, which use microtubules for inward transport and microtubules/actin for egress, both the retrograde (transport towards nucleus) and anterograde transport (egress of progeny virions) of baculovirus are dependent on the actin cytoskeleton for multiple steps (Fig. 9.4A). Thus,

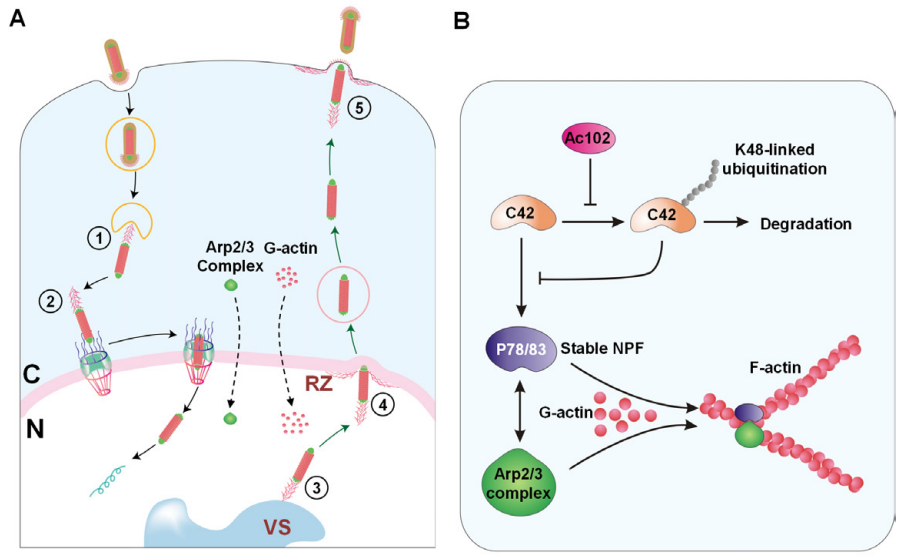


**Figure 9.4** Baculovirus-induced actin polymerization. (A) The multiple functions of actin polymerization during baculovirus infection. After penetration of the nucleocapsids into the cytoplasm, actin polymerization is immediately induced for transport of nucleocapsids towards the nucleus (step ①) and passage through NPC (step ②). Within the infected nucleus, the nuclear F-actin network transports progeny nucleocapsids from the virogenic stroma (VS) to the nuclear periphery (ring zone, RZ) (step ③) and further provides the driving force for nucleocapsid egress from the nucleus (step ④). In the cytoplasm, F-actin is again exploited for the trafficking of nucleocapsids to the plasma membrane for further BV budding (step ⑤). (B) The regulatory mechanism of baculovirus-induced actin polymerization. Ac102 suppresses K48-linked ubiquitination and proteasomal degradation of C42, thus allowing P78/83 to function as a stable nucleation promoting factor (NPF) to interact with host Arp2/3 complex for actin nucleation and polymerization. C, cytoplasm; N, nucleus.

baculoviruses appear to have developed a unique and dedicated regulatory mechanism for usurping the cellular actin cytoskeleton for virus morphogenesis and intracellular transport (Fig. 9.4B).

## Retrograde cytoplasmic transport of nucleocapsid

The dynamic transition between globular actin (G-actin) monomers and filamentous actin (F-actin) forms for actin cytoskeleton remodelling is critical for diverse cellular activities. Actin polymerization is required for transport of nucleocapsids to the nucleus where virus replication takes place (Fig. 9.4A, step ①). Immediately following penetration of the nucleocapsids into the cytoplasm (approximately 30 min p.i.), thick actin cables are induced (Charlton and Volkman, 1993). Conversely, actin cable formation was not observed when virus–endosome fusion was inhibited by drugs, suggesting that nucleocapsid proteins induce the polymerization of actin (Charlton and Volkman, 1993). Ohkawa *et al.* (2011) demonstrated that AcMNPV undergoes actin-based motility in the cytoplasm, colliding with and sticking to the nuclear envelope, which could be blocked by an actin-stabilizing drug (Ohkawa *et al.*, 2010). In eukaryotic cells, the actin polymerization process normally requires an N-Wiskott-Aldrich syndrome protein (N-WASP) acting as the nucleation promoting factor (NPF). N-WASP activates the actin-related protein 2/3 (Arp2/3) complex to promote G-actin nucleation and form F-actin. The baculovirus genome encodes an N-WASP-like protein, P78/83, which localizes to the blunt end of the nucleocapsid. P78/83 activates the host Arp2/3 complex for actin polymerization (Goley *et al.*, 2006). Electron tomography showed that the AcMNPV nucleocapsid was propelled by continuously assembled actin comet tails, with an average of four fishbone-like actin filaments per nucleocapsid (Mueller *et al.*, 2014).

## Virus passage through the nuclear pore complex

It is now generally accepted that baculoviruses enter the nucleus via the nuclear pore complex (NPC), although the entry modes exploited may vary among different baculoviruses (Fig. 9.4A, step ②). For betabaculoviruses, early electron microscopy studies observed the attachment of empty nucleocapsids to the cytoplasmic side of the NPC and the absence of intact nucleocapsids in the nucleus, suggesting that virus uncoating took place at the NPC (Summers, 1969, 1971). In contrast, alphabaculoviruses appear to traverse the NPC with intact nucleocapsids, both in insect and mammalian cells (Granados and Lawler, 1981). AcMNPV nuclear entry was suggested to be dependent on NPC based on the use of a truncated form of importin-β or wheat germ agglutinin (a nuclear transport inhibitor) to interfere with the function of NPC (Ohkawa *et al*., 2010). Using microinjection of *Xenopus* oocytes and electron tomography, Au *et al*. (2013) clearly visualized an AcMNPV capsid vertically docking at the cytoplasmic side of NPCs with its apical cap end, crossing the NPC, and finally reaching the nucleus in intact form (Au and Panté, 2012). During this process, the NPC central channel switched from a 'closed' to an 'open' state to allow the passage of the nucleocapsids (Au and Panté, 2012; Au *et al*., 2013).

The nuclear import of large cargoes (proteins larger than 40 kDa or molecules larger than 9 nm in diameter) requires the recognition of special signals within the cargoes by cellular nuclear import receptors (importins or karyopherins), and further translocation of the cargoes through the NPC (Cohen *et al*., 2011). To date, the nuclear import mechanism of baculovirus nucleocapsids remains unclear. Two viral nucleocapsid proteins, Ac109 and Ac132, may be involved in the entry of viral nucleocapsids into the nucleus (Alfonso *et al*., 2012; Fang *et al*., 2016). Deletion of either gene produces non-infectious BVs that are able to enter the cytoplasm but fail to enter the nucleus. However, both of the proteins lack a predicted nuclear localization signal (NLS), therefore being unlikely to directly interact with classical cellular nuclear transport receptors. It is also not known whether Ac109 and Ac132 are involved in the interaction between nucleocapsids with NPC or have a role in a step following NPC docking. Another viral nucleocapsid protein, VP80, is hypothesized to play a role in mediating the nuclear import of nucleocapsids because it is the only viral nucleocapsid protein containing a predicted NLS, although this requires further investigation (Au *et al*., 2013).

An alternative unconventional nuclear import mechanism was recently proposed for AcMNPV. Using a well-established nuclear import assay, the nuclear entry of AcMNPV was found to be independent of importin β and Ran-GTPase used in the classical nuclear import pathway. The nuclear import of nucleocapsids was recreated instead under conditions supporting actin polymerization, suggesting that F-actin not only directs the motility of nucleocapsids towards the nuclear periphery within the cytoplasm, but also provides the driving force for the translocation of nucleocapsids through the NPC (Au *et al*., 2016).

## Nuclear localization of G-actin and Arp2/3 complex

The nuclear localization of G-actin (NLA) is mediated by six AcMNPV early genes (*ie-1*, *pe38*, *he65*, *ac004*, *ac102*, and *ac152*) as shown via transient transfection assay (Ohkawa *et al*., 2002). Among these genes, only *ac102* is essential, albeit not sufficient for mediating NLA. Co-transfection of an *ac102*-deleted bacmid with a plasmid expressing NLS-GFP-actin did not rescue infectious progeny BV production, suggesting that Ac102 has other essential roles beyond mediating NLA (Gandhi *et al*., 2012). Based on evidence that an NLA-blocking drug inhibits late gene expression and the deletion of *ac102* does not have an obvious impact on actin nuclear accumulation, an AcMNPV late gene other than *ac102* could play a key role in mediating NLA (Zhang, Y. *et al*., 2018). In turn, the nuclear accumulation of the Arp2/3 complex is facilitated by a viral encoded late protein, Ac34. This protein inhibits the CRM1-dependent nuclear exportin pathway, leading to the retention of Arp2/3 in the nucleus to promote actin polymerization (Mu *et al*., 2016a,b).

## Anterograde transport of nucleocapsid

A unique feature of baculovirus infection is that substantial nuclear actin polymerization is induced at the late stage of infection and plays a critical role in progeny virus morphogenesis (Ohkawa and

Volkman, 1999; Goley *et al.*, 2006). The nuclear F-actin cytoskeleton interacts with the virus-encoded motor molecule VP80 to form a network for transport of progeny nucleocapsids from the virogenic stroma (VS) to the nuclear periphery (ring zone, RZ) (Marek *et al.*, 2011) (Fig. 9.4A, step ③). Further nuclear egress is also dependent on actin polymerization, including the direction of nucleocapsids into nuclear envelope protrusions and local disruption of nuclear envelope integrity (Ohkawa and Welch, 2018) (Fig. 9.4A, step ④). Notably, AcMNPV egress from the nucleus to the cytoplasm via NPC and by the classical nuclear export pathway was excluded by using specific inhibitors (Ohkawa and Welch, 2018). AcMNPV again exploits F-actin in the cytoplasm, likely together with microtubules, for the trafficking of nucleocapsids to the plasma membrane for further BV budding (Ohkawa *et al.*, 2010; Ohkawa and Welch, 2018) (Fig. 9.4A, step ⑤).

### Regulatory mechanism of baculovirus-induced actin polymerization

Baculovirus-induced actin polymerization is orchestrated by G-actin, the nucleator Arp2/3 complex, and the virus NPF P78/83 (Goley *et al.*, 2006). AcMNPV has developed a sophisticated regulatory cascade to control the activity of viral NPF P78/83 for nuclear actin polymerization. BV/ODV-C42 (C42) is responsible for recruiting P78/83 to the nucleus (Wang, Y. *et al.*, 2008). C42 also protects P78/83 from ubiquitin-independent proteasomal degradation by masking a degron in P78/83, thus ensuring its NPF activity in mediating actin polymerization (Li *et al.*, 2010; Wang *et al.*, 2015). Ac102 suppresses ubiquitination and proteasomal degradation of C42, thus allowing P78/83 to function as a stable NPF to induce actin polymerization (Hepp *et al.*, 2018; Zhang, Y. *et al.*, 2018). A dynamic regulatory mechanism for P78/83-induced actin polymerization and nucleocapsid decapsidation has been proposed. This includes the degradation of P78/83 by exposing the degron for detachment of F-actin from the nucleocapsids and further release of the viral genome in the nucleus, as well as the stabilization of P78/83 by C42 to facilitate nuclear actin polymerization allowing progeny virus morphogenesis and transport (Wang *et al.*, 2015) (Fig. 9.4B).

## Genome replication

Virus genome replication begins on entry into the nucleus. As a large DNA virus, the replication of baculovirus presents a complex picture. Viral genomes usually contain multiple separate origins of replication for initiation of DNA synthesis, which may ensure replication efficiency. Baculovirus genomes also encode a complicated and dedicated replication machinery that creates a factory within the infected nucleus dedicated to viral genome replication.

### Baculovirus origin of replication

Two different approaches, generation of defective genomes and replication assays, have been employed to identify viral origins of DNA replication. In the former, BV is continuously passaged at high multiplicity of infection, resulting in the enrichment of defective interfering (DI) particles with much smaller genome sizes. The generation of defective genomes with enriched *hr* sequences, implicates them as origins of replication (Pearson *et al.*, 1992; Kool *et al.*, 1993). In replication assays, plasmids containing various *hrs* are transfected into virus-infected cells, and newly synthesized DNA is distinguished from input DNA by *Dpn* I digestion. Using this approach, all eight *hr*s of AcMNPV have shown the capacity to act as origins of replication (Kool *et al.*, 1993; Leisy and Rohrmann, 1993). Similar methods have also identified non-*hr* sequences and a variety of early gene promoters as origins of replication (Lee and Krell, 1992; Pearson *et al.*, 1993; Wu *et al.*, 1999). Recently, a novel replication origin within the *p143* (helicase) coding sequence was identified to replicate more efficiently in insect cells than the known *hr*- or non-*hr* origins and could function even in mammalian cells (Wu *et al.*, 2014). It has been suggested that DNA replication could initiate at any unwound sequences that allow for entry of the replication complex. Deletion of individual *hr* sites did not have an obvious impact on AcMNPV replication and simultaneous elimination of all eight *hrs* did not prevent the production of infectious progeny virus, although virus titre was reduced (Carstens and Wu, 2007; Bossert and Carstens, 2018). However, some degree of viral-host specificity for *hr* origins does appear to exist, as evidenced by the origin of one virus replicating poorly in cells infected by another virus (Pearson *et al.*, 1993). Thus, the presence of redundant origins

of replication in the baculovirus genome may provide functional complementarity and increase the speed of the virus replication cycle.

## Viral genes involved in DNA replication

Six genes essential for DNA replication have been identified using a transient replication assay with overlapping cosmid clones encompassing the complete genome of AcMNPV (Kool *et al.*, 1994). These include genes for DNA polymerase (*dnapol*), helicase (*p143*), *lef-1*, *lef-2*, *lef-3*, and *ie-1*. The first four are core genes of baculoviruses (Table 9.1). *In vivo* study confirmed that *dnapol* (Vanarsdall *et al.*, 2005), *p143* (Lu and Carstens, 1991), *lef-3* (Yu and Carstens, 2010), as well as a new member, *lef-11* (Lin and Blissard, 2002), are essential for DNA replication.

### DNA polymerase

The baculovirus DNA polymerase is in the DNA polymerase B family (Tomalski *et al.*, 1988; Braithwaite and Ito, 1993). It also exhibits a 3′ to 5′ exonuclease activity for proofreading of mispaired nucleotides (Hang and Guarino, 1999). In AcMNPV infected cells, the transcription of *dnapol* was detected in a narrow period between 2–8 h.p.i. with peak at 4–6 h.p.i (Tomalski *et al.*, 1988). The coding sequence of AcMNPV DNA polymerase contains 948 aa with an exonuclease domain (aa 185–401), a polymerase domain (aa 510–750), and two C-terminal NLSs (Feng and Krell, 2014). The N-terminal region (aa 1–185) of AcMNPV DNA polymerase is also conserved in other baculovirus DNA polymerase sequences and is essential for DNA replication (Chen *et al.*, 2018).

### Helicase

Helicase (P143) of baculovirus constitutes a motor protein that unwinds DNA by disrupting the hydrogen bonds between bases in the DNA double helix. It contains both ATPase and helicase activity and can bind non-specifically to single-stranded DNA (ssDNA) and dsDNA (Lu and Carstens, 1991; McDougal and Guarino, 2000). An inchworm DNA unwinding mechanism has been proposed for baculovirus helicase wherein the protein binds to dsDNA with high affinity, then the hydrolysis of ATP causes a conformational change of the protein leading to its disassociation from DNA. Through cycles of ATP hydrolysis and helicase translocation, dsDNA is unwound (McDougal and Guarino, 2001). Helicase interacts with LEF-3 and depends on the latter to be transported into the nucleus (Wu and Carstens, 1998). Notably, mutation of two key residues in the helicase sequence expands the host range of an AcMNPV isolate to *Bombyx mori*, an original non-permissive host for that strain of AcMNPV (Argaud *et al.*, 1998).

### LEF-1

LEF-1 contains a conserved primase domain (WVVDAD), and purified LEF-1 exhibits primase activity (Mikhailov and Rohrmann, 2002). Although LEF-1 shows low affinity for ssDNA, it interacts with LEF-2 (Evans *et al.*, 1997) and co-purified LEF-1 and LEF-2 exhibit high affinity for ssDNA (Mikhailov and Rohrmann, 2002). *Lef-1* appears to be an essential gene in BmNPV (Ono *et al.*, 2012), but its requirement for viral DNA replication *in vivo* has not been confirmed.

### LEF-2

LEF-2, a primase accessory factor, interacts with both LEF-1 and DNA and may stabilize LEF-1 binding to DNA (Mikhailov and Rohrmann, 2002). LEF-2 serves as a capsid protein of both BV and ODV, suggesting that the presence of LEF-2 immediately after entry is important for DNA replication (Wu *et al.*, 2010). Deletion of *lef-2* showed that it is essential for viral DNA amplification but not for initiating DNA replication (Wu *et al.*, 2010). LEF-2 is also involved in late gene expression (Passarelli and Miller, 1993; Merrington *et al.*, 1996).

### LEF-3

LEF-3 constitutes an ssDNA binding protein (SSB) (Hang *et al.*, 1995) that is conserved in lepidopteran baculoviruses but is not found in gamma- and delta baculoviruses. LEF-3 interacts with and mediates nuclear import of the helicase (Wu and Carstens, 1998); consistent with this, a knock-out study showed that LEF-3 is essential for viral DNA replication *in vivo* (Yu and Carstens, 2010). Specifically, LEF-3 forms homo-oligomers that may facilitate its function (Mikhailov *et al.*, 2006; Downie *et al.*, 2013) and forms a complex with alkaline nuclease (AN) that exhibits 5′–3′ exonuclease activity (Mikhailov *et al.*, 2003).

### IE-1

IE-1 is an immediate early gene that binds to *hr*s as a transcriptional activator (Choi and Guarino, 1995; Rodems and Friesen, 1995). The N-terminal 2–23 aa of AcMNPV IE-1 are critical for DNA replication. This domain contains a consensus cyclin-dependent kinase phosphorylation site, the mutation of which results in the loss of viral DNA replication. This suggests that phosphorylation of IE-1 may be involved in recruiting replicative factors to the *hr* origins of DNA replication (Taggart *et al.*, 2012).

### LEF-II

LEF-11 is conserved at the gene level in all baculoviruses except *Culex nigripalpus nucleopolyhedrovirus* (CuniNPV, deltabaculovirus) and has been identified as essential for DNA replication via knock-out assay *in vivo* (Lin and Blissard, 2002). In BmNPV, LEF-11 co-localizes with IE-1 (Zhang *et al.*, 2014), with oligomerization apparently critical for DNA replication (Dong, Z.Q. *et al.*, 2015). LEF-11 also interacts with two host ATPase family members, ATAD3A and HSPD1 (HSP60), to facilitate virus manipulation (Dong *et al.*, 2017). Whether this is related to the function of LEF-11 in DNA replication requires further investigation.

Other baculoviral genes are also involved in baculovirus DNA replication, including a gene encoding for DNA binding protein (DBP) (Vanarsdall *et al.*, 2007a), *me53* (Xi *et al.*, 2007) and *lef-7* (Lu and Miller, 1995).

## The virogenic stroma

Baculovirus infection induces a special subnuclear structure, termed the virogenic stroma (VS), that consists of a network of electron-dense cords with electron-translucent spaces sensitive to salt and RNase (Young *et al.*, 1993). The VS constitutes the factory for viral DNA replication (Kawasaki *et al.*, 2004). Viral proteins involved in DNA replication, including IE-1, DBP, LEF-3, and PP31 (39K), localize to the VS, with the sizes of the foci dramatically increasing during infection to occupy over half of the nucleoplasm (Guarino *et al.*, 1992; Okano *et al.*, 1999). LEF-2 also localizes to the VS (Wu *et al.*, 2010). Inhibition of viral DNA replication by aphidicolin or deletion of *dbp* disturbs the proper formation of the VS (Vanarsdall *et al.*, 2007a). Co-expression of four viral proteins including IE-1, LEF-3, P143, and PP31 is sufficient to induce the formation of a cellular chromatin-containing VS-like structure in the nuclei of uninfected cells (Nagamine *et al.*, 2011). The VS also serves as the factory for transcription and nucleocapsid assembly.

## Viral gene transcription

Baculovirus gene transcription is divided into early and late (including late and very late) stages separated by the onset of DNA replication. Two distinct RNA polymerase machineries encoded by the host or the virus itself synthesize early- and late/very late-mRNAs, respectively. These two RNA polymerase complexes recognize different promoter motifs and directly program viral gene expression.

## Early gene transcription

Early genes are transcribed by host RNA polymerase II, which is sensitive to alpha amanitin. Early gene products are generally required for initiating DNA replication or executing other early events, including factors for viral replication, GP64 for establishing early systemic infection and P35 for blocking host apoptotic response. Both transcriptional enhancers (*hrs*) and activators play a major role in early transcription.

### IE-1

IE-1, in addition to being essential for DNA replication, is the major activator required for early gene transcription. It contains an N-terminal transcription activation domain and a C-terminal DNA binding domain (Kovacs *et al.*, 1992). IE-1 forms dimers and binds to the palindromes of *hrs* in the absence of other proteins, leading to elevation of the transactivation activity of IE-1 as much as 200-fold. A helix–loop–helix domain at aa 543–568 of AcMNPV IE1 mediates dimerization and is required for *hr* binding (Olson *et al.*, 2001). A conserved basic domain at aa 152–161 is the enhancer binding domain for IE1 transcription activation (Olson *et al.*, 2003). IE-1 can also activate transcription independent of *hrs*, most likely by directly interacting with the transcription complex.

### IE-0

IE-0 is structurally identical to IE-1, except it contains an additional 54 aa at its N-terminus. IE-0

is produced by splicing a segment of the exon0 transcript encoding 38 aa to the 5′ untranslated region of the *ie-1* mRNA (encoding 16 aa and the entire IE-1). Both IE-0 and IE-1 can regulate transcription of early- and late-genes. IE-1 can also down-regulate transcription under the control of the *ie-0* promoter. IE-0 expression peaks at the very early stage of infection and declines thereafter, while IE-1 becomes more abundant than IE-0 after the onset of DNA replication. Some viral promoters are preferably transactivated by IE-0 to IE-1, suggesting that at the very early stage of infection certain genes are specifically regulated by IE-0 (Sokal *et al.*, 2014).

### IE-2

IE-2, an additional transcriptional activator that occurs only in some Group I alphabaculoviruses, contains a predicted RING finger domain that can augment IE-1-mediated transactivation when co-transfected with IE-1 (Carson *et al.*, 1988; Yoo and Guarino, 1994). The transactivation effect of IE-2 appears to be cell-line specific or host-specific (Lu and Miller, 1995; Prikhod'ko *et al.*, 1999). IE2 appears to have ubiquitin-ligase (E3) activity (Imai *et al.*, 2003), whether this is related to its transactivation function still awaits further investigation.

## Late gene transcription

In contrast to early gene transcription, late/very late genes are transcribed by virus-encoded transcription factors and thus are insensitive to amanitin. Late transcription likely starts after DNA replication, based on the fact that inhibitors of DNA replication block late gene transcription. The results of transient expression assays identified 19 genes involved in late gene transcription, known as late expression factors (*lefs*) (Rapp *et al.*, 1998). Ten of these are required for optimal DNA replication and are known as replication *lef* genes (Kool *et al.*, 1994; Lu and Miller, 1995). The remaining nine genes, including *lef4–6*, *lef8–10*, *lef-12*, *p47* and *39k*, are termed transcription *lef* genes.

In 1998, a virus-encoded RNA polymerase complex that can support transcription from late/very late promoters was purified from AcMNPV-infected cells (Guarino *et al.*, 1998). This approximately 560 kDa protein complex consists of four subunits: LEF-4 (54 kDa), LEF-8 (102 kDa), LEF-9 (55 kDa), and P47 (47 kDa), markedly simpler than bacterial and eukaryotic RNA polymerases. The four subunits are present in almost equimolar amounts, so this complex may contain two copies of each protein. Overexpression of the four subunits significantly increases transcriptional activity at the late stage of infection (Guarino *et al.*, 1998).

### LEF-4

LEF-4 constitutes a metal-dependent mRNA capping enzyme. In eukaryotes, three enzymatic activities are needed for cap formation: an RNA triphosphatase to hydrolyse the 5′-triphosphate end of mRNA to a diphosphate, an RNA cap guanylyltransferase to cap the diphosphate end with GMP, and an RNA cap methyltransferase to catalyse N-7 methylation of the guanine cap. LEF-4 contains both triphosphatase and guanylyltransferase activities, located in the N-terminal and C-terminal region of the protein, respectively (Gross and Shuman, 1998; Jin *et al.*, 1998; Martins and Shuman, 2003). The guanylyltransferase function but not the RNA triphosphatase function of LEF-4 is essential for productive infection (Knebel-Mörsdorf *et al.*, 2006; Li and Guarino, 2008).

### LEF-8

LEF-8 is the largest subunit of the baculovirus RNA polymerase complex and contains a conserved motif, $GXKX_4HGQ/NKG$, which is found in the β or β' subunit of DNA-directed RNA polymerases from a wide range of organisms (Passarelli *et al.*, 1994). This motif is generally located at the C-terminus of the enzyme and comprises part of the catalytic site. Mutagenesis analysis shows that the conserved C-terminal motif is critical for late gene expression (Titterington *et al.*, 2003). *lef-8* is considered an essential gene for BV production/late gene transcription (Gauthier *et al.*, 2012; Ioannidis *et al.*, 2016).

### LEF-9

LEF-9 contains a 7-aa motif (NTDCDGD) similar to the $Mg^{2+}$ binding sequence (NADFDGD) that comprises the catalytic domain present in the largest subunits of some RNA polymerases. This motif

is essential for LEF-9 mediated late gene expression (Crouch *et al.*, 2007). *lef-9* is considered an essential gene, as its deletion in BmNPV fails to produce infectious progeny virus (Gomi *et al.*, 1997; Ono *et al.*, 2012).

### P47

P47 was originally identified via a *ts* mutant that caused a severe reduction in late gene expression (Partington *et al.*, 1990; Carstens *et al.*, 1993). This protein shows low sequence similarity to the α subunit of bacterial RNA polymerase, which is involved in the initiation of RNA polymerase assembly (Rohrmann, 2013). The exact role of P47 in the RNA polymerase complex has yet to be determined.

### LEF-5

LEF-5 is conserved in all sequenced baculoviruses. LEF-5 strongly stimulates transcription activity of purified baculovirus RNA polymerase complex (Guarino *et al.*, 2002). LEF-5 interacts with itself (Harwood *et al.*, 1998) and is essential for productive infection (Su *et al.*, 2011). It contains a motif similar to the zinc ribbon domain of eukaryotic RNA polymerase II elongation factor SII, deletion of which resulted in a 70% reduction in LEF-5 activity (Harwood *et al.*, 1998). An *in vitro* activity assay indicates that LEF-5 may not act as an elongation factor as SII, but rather play a role in the transcription initiation (Guarino *et al.*, 2002).

### VLF-1

VLF-1 (very late expression factor 1), a member of the lambda integrase family encoded by the core gene *vlf-1*, is required for high-level expression of very late genes (polyhedrin (*polh*) and *p10*) (McLachlin and Miller, 1994). The promoters of the very late genes differ from those of the late genes; very late genes possess an A+T rich burst sequence downstream of the transcription initiation site. *In vitro* transcription assays show that VLF-1 can stimulate transcription by binding to these 'burst' sequences in very late promoters (Yang and Miller, 1998; Mistretta and Guarino, 2005). A mutation from cysteine to a tyrosine at residue 202 appears to abolish the activity of AcMNPV VLF-1 (McLachlin and Miller, 1994). VLF-1 is also involved in processing of the nucleocapsid assembly and DNA packaging (Vanarsdall *et al.*, 2006).

## Global patterns of gene transcription

A comprehensive single nucleotide resolution analysis to globally map transcription start sites (TSS), polyadenylation sites (PAS), and mRNA abundance of the AcMNPV transcription has been performed in cultured *Trichoplusia ni* cells (Chen *et al.*, 2013). A total of 218 TSS for 156 AcMNPV ORFs were identified, with 46 ORFs containing multiple TSS. Among these, 126 TSS for 101 genes (65%) were mapped at the consensus later promoter motifs (TAAG); the remaining 92 TSS associated with 77 genes (49%) were non-TAAG sites, thus being likely to generate early transcripts. Twenty-one genes (13%) appear to contain both early and late promoters. For the 126 identified TAAG motifs, transcription exclusively initiated from the second position (T<u>A</u>AG, underlined). The sequences of non-TAAG motif-containing TSS were much more diverse. Only 29 of the 92 TSS contained upstream TATA boxes and 14 initiated within or near the previously described CAGT start site, although 84% initiated at an A residue. The average 5′-UTR lengths of early (non-TAAG motif) and late (TAAG motif) genes were 155 and 169 nt, respectively.

Analysis of 3′PAS identified a total of 120 PAS corresponding to 135 ORFs. Moreover, 16 genome regions were likely to terminate at a common PAS. The majority (approximately 77%) of the identified PAS were located within 30 nt downstream of a consensus termination signal (AUUAAA/AUUAAA). The average length from the stop codon to the downstream of PAS was 338 nt. In 58 transcripts, a U-rich region was located 2–10 nt downstream of the PAS.

Temporal gene expression was analysed by strand-specific RNA sequencing (RNA-Seq). At 6 h.p.i. the majority of transcripts were early genes, including those involved in DNA replication (*lef-2*, *lef-3*, *dbp*, and *pp31*) and anti-apoptosis (*p35*), as well as the major EFP *gp64*. From 6–12 h.p.i. virus transcription was highly activated, with virus mRNA increasing from 3% (6 h.p.i.) to 38% (12 h.p.i.) of the total cellular mRNA. This may be associated with the onset of virus DNA replication. At 12 h.p.i. 109 genes were transcriptionally active; the most abundant of which encode virus structural proteins (*p6.9*, *odv-ec27*, and *odv-e18*). From 24 h.p.i., *polh* and *p10* were the most abundant mRNAs, accounting for 24 and 7.5% of the total

cellular mRNA. At the late stage of infection, viral transcripts comprised approximately 80% of the total mRNA of infected host cells. Overall, genes with early promoters peaked at 6–12 h.p.i., whereas those containing late promoters were expressed at the highest levels at 12–18 h.p.i. or later. Interestingly, low levels of mRNA of 40 genes were already present at 0 h.p.i. As most of these show highly abundant late transcripts, these late mRNAs may be incorporated into BVs during the nucleocapsid assembly and budding process. Twelve spliced transcripts (including the previously characterized *ie-0*) and antisense RNAs to 50 genes were also identified, suggesting possibly significant roles in regulating AcMNPV gene expression.

A transcriptomic analysis of AcMNPV gene expression in the midgut of *T. ni* larvae was published by the same research group (Shrestha *et al*., 2018). Viral gene expression profiles in ODV-infected midgut share marked similarities with those in BV-infected cell lines. However, several genes associated with enhanced F-actin mediated viral motility (*arif-1* and *ac102*), accelerated systemic infection (*v-fgf*), DNA replication (*lef-3* and *an*) and nucleocapsid assembly (*ac54*) were expressed at much higher levels in the midgut than in cell lines. In contrast, *fp-25k*, a negative factor for BV production, was expressed at a lower level in the midgut. These differential expression patterns represent specific viral adaptations for rapid egress from the midgut to disseminate systemic infection.

## Nucleocapsid assembly

The next stage of virus infection is the assembly of progeny nucleocapsids, followed by the formation of BVs. Baculovirus nucleocapsids (30–60 nm × 250–300 nm) are characterized by cylindrical capsids with distinct apical cap and basal structures, with an electron-dense internal nucleoprotein core. In AcMNPV, 24 proteins are associated with the nucleocapsids of both BV and ODV phenotypes, including 11 core proteins (Table 9.1) (Blissard and Theilmann, 2018). Nucleocapsid assembly is a highly organized (both in time and space) and complex process. A brief model for baculovirus nucleocapsid assembly has been proposed where viral genomic DNA is condensed by viral proteins and the resulting nucleoprotein core is packaged into pre-assembled empty capsids (Fig. 9.5).

### Nucleocapsid morphogenesis

The progeny nucleocapsids dispersed within the VS were first observed at 8 h.p.i. in the midgut epithelial cells of *T. ni* larvae infected with AcMNPV (Granados and Lawler, 1981). The kinetics of virus replication and morphogenesis appears to be slower in AcMNPV-infected *Mamestra brassicae* cells, with formation of VS observed at 7 h.p.i., empty capsids appearing at 10 h.p.i. and partially or fully filled nucleocapsids observed at 10–11 h.p.i. (Bassemir *et al*., 1983) A low-resolution structural model of the nucleocapsid of *Spodoptera litura granulovirus* (SpliGV) was created based on electron microscopy and x-ray scattering in 1982. The cylindrical body of the viral capsid is composed of a 12-part helix of monomers. Structural proteins, which form caps at both ends of the nucleocapsid, appear to be distinct from those constituting the cylindrical body. Within the nucleocapsid, the dsDNA associates with a highly basic protein (P6.9) to form a cylindrical core (Burley *et al*., 1982). An investigation of AcMNPV genome packaging and virion morphogenesis in Sf9 cells showed the formation of empty capsids of nearly full length, most likely initiating with the basal structures; the association of these empty capsids with their apical cap ends to the matrix of VS, composed of newly synthesized DNA and nucleoproteins; and the packaging of the nucleoprotein core from the base to the apex of the capsid sheath to form the filled nucleocapsids, apparently directed by the apical cap structure (Fraser, 1986). Recent cryo-EM analysis of AcMNPV BV confirms earlier observations that the helical structure of nucleocapsids contains a high electron density core with an apical nipple-like structure and a basal claw-like structure at different ends. At least three viral proteins, including P78/83, VLF-1 and VP80, are associated with the basal end region (Rohrmann, 2013). Empty capsids possess a rigid tubular structure and share identical shape and size with filled nucleocapsids, suggesting that viral capsid assembly is independent of DNA packaging (Wang *et al*., 2016). This independence is further supported by the discovery that long, branched tubules were common in *Trichoplusia ni granulovirus* (TnGV)-infected *T. ni* larvae; these may be self-assembled capsid proteins (Summers, 1971). The elimination of genes such as *p6.9* (Wang *et al*., 2010a), *38k* (Wu *et al*., 2006), *vlf-1* (Vanarsdall *et al*., 2006), *vp1054* (Guan *et al*., 2016), *p78/83* (Li *et al*., 2010), *c42* (Li

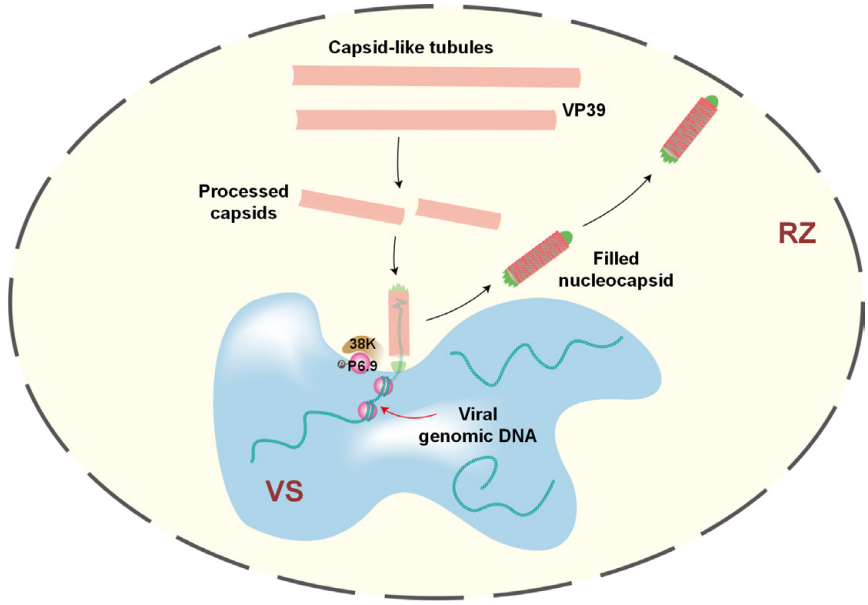


**Figure 9.5** Simplified model for baculovirus nucleocapsid assembly within infected nucleus. Capsid-like tubules formed by VP39 are processed into empty capsids. The viral DNA binding protein P6.9 is dephosphorylated by viral phosphatase 38K, allowing P6.9 to bind and condense newly synthesized viral genomic DNA. Subsequently, the nucleoprotein core is packaged from the base to the apex of the pre-assembled capsid sheath to form filled nucleocapsids. Nucleocapsids are then assembled in the virogenic stroma (VS) and filled nucleocapsids are transported to the ring zone (RZ) to form BV or ODV.

*et al.*, 2010; Vanarsdall *et al.*, 2007b), *ac53* (Liu *et al.*, 2008), *ac142* (Vanarsdall *et al.*, 2007b) and *ac144* (Vanarsdall *et al.*, 2007b) resulted in accumulation of these elongated, empty capsid-like tubules. Therefore, the intact capsid sheath may represent the processed product of these long tubules, with capsid assembly occurring prior to DNA packaging, rather than simultaneously. The insertion of an ≈15 kb DNA fragment into the genome of AcMNPV led to the increase of capsid length by about 13 nm (from 260 to 283 nm) (Fraser, 1986). Furthermore, the capsid length was shorter with defective genomes (Kool *et al.*, 1991). In combination, this suggests that the length of the baculovirus capsid is flexible in response to genome size.

## Key proteins involved in nucleocapsid assembly

During the process of nucleocapsid assembly, a virus-encoded protamine-like DNA binding protein (P6.9) condenses newly synthesized viral DNA ≈100-fold, followed by packaging into the pre-assembled capsids made of the major capsid protein VP39 and other minor capsid proteins.

### VP39

VP39 is the most abundant viral capsid protein with homologues in all sequenced baculovirus genomes. Immunoelectron microscopy analysis showed that the electron-lucent capsid-like tubular structures reacted specifically with a VP39 antibody (Vanarsdall *et al.*, 2006; Wu *et al.*, 2006). Consistent with this, when VP39 of HearNPV was expressed using a prokaryotic system, it was able to self-assemble into viral capsid-like helical tubes *in vitro* (Rao *et al.*, 2018), indicating VP39 has a major role in forming viral capsid structures. Specifically, a conserved glycine residue (Gly-276) of VP39 appeared to be essential for nucleocapsid assembly and very late gene expression (Katsuma and Kokusho, 2017).

Recently, Cryo-EM tomography was used to construct a higher resolution 3D structure of the HearNPV VP39 helical tubes. Two slightly different structural forms were observed: a narrow (N)-tube type (the major type, comprising approximately 90%) and a wide (W)-tube type (the minor type, approximately 10%). The diameters of the two types (25 nm for N-type and 28 nm for W-type) of helix tubes are close to the size of the native

capsids. N-tubes are organized as a continuous helix composed of 11⅓ subunits per turn, whereas W-tubes are arranged as ring-shape building blocks with 12 subunits per turn (Fig. 9.6). The structure of the W-tube type is consistent with the previously proposed nucleocapsid model of SpliGV (Burley *et al.*, 1982). These data provide an important basis for understanding the mechanism of baculovirus nucleocapsid assembly and supports the application of VP39-based novel nanomaterials for loading functional motifs (Rao *et al.*, 2018).

## P6.9

P6.9 is a virus encoded DNA binding protein found in all sequenced baculoviruses. P6.9 is a small basic protein rich in arginine, serine, and threonine responsible for binding and condensing viral DNA for genome encapsidation. P6.9 is highly abundant in the nuclear matrix and is believed to replace host cellular histones from newly synthesized DNA prior to genome packaging, as it is considerably more basic than host histones (Wilson and Miller, 1986). P6.9 is a hyper-phosphorylated protein, with 22 phosphorylation sites identified by mass spectrometry (Li *et al.*, 2015). The C-terminal basic domain plays a key role in nucleocapsid assembly and specificity (Wang *et al.*, 2010a). Notably, the phosphorylation state of P6.9 is under dynamic regulation in different stages of the virus life cycle. Distinct forms of P6.9 phosphorylated at different sites were identified in infected cells, whereas only dephosphorylated P6.9 was found in BVs (Liu *et al.*, 2012). During nucleocapsid uncoating, P6.9 is phosphorylated by the viral protein kinase PK1 and potentially other host kinases to dissociate from viral DNA, thus facilitating the release of the viral genome (Li *et al.*, 2015). In contrast, upon nucleocapsid assembly at VS, P6.9 is dephosphorylated by 38K to promote DNA binding (Lai *et al.*, 2018).

## 38K

The protein 38K is a nucleocapsid protein distributed over the cylindrical capsid sheath of both BV and ODV (Wu *et al.*, 2008). 38K is conserved in baculovirus, nudivirus, and polydnavirus genomes. Deletion of the AcMNPV *38K* gene led to the formation of aberrant, elongated, empty capsid-like tubules (Wu *et al.*, 2006). Lai *et al.* (2018) suggested that 38K was a member of the viral phosphatase superfamily and identified P6.9 as one of its substrates. The aa 135–288 of AcMNPV 38K contains haloacid dehalogenase (HAD) motifs that are involved in phosphoryl transfer. Mutating the key amino acids of these motifs resulted in empty tube-like structures in the VS and absence of nucleocapsids. A functional study suggested that 38K was involved in genome encapsidation by mediating the dephosphorylation of five phosphorylated sites at the C terminus of P6.9 (Lai *et al.*, 2018). 38K may have other functions based on its interaction with multiple viral capsid proteins, including VP39, VP80, and VP1054 (Wu *et al.*, 2008).

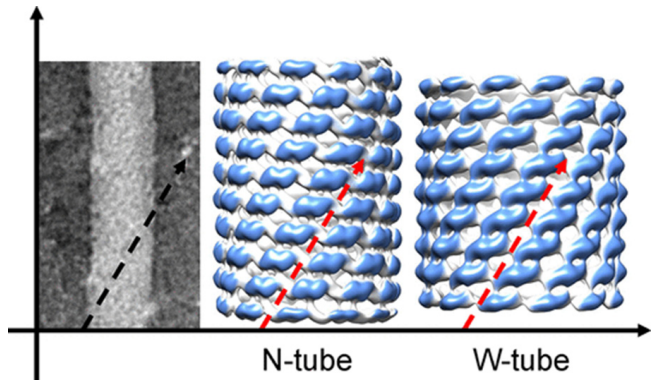


**Figure 9.6** Cryo-EM reconstructions of HearNPV VP39 N-tubes and W-tubes. Cryo-EM tomography was used to reconstruct 3D structures of the HearNPV VP39 helical tubes (left). Two slightly different structural forms, the narrow (N)-tube (25 nm in diameter, the major type) (middle) and wide (W)-tube (28 nm in diameter, the minor type) (right) were detected. N-tubes are organized as a continuous helical line wraps around with $11^{1}/_{3}$ subunits per turn, whereas W-tubes are arranged as ring-shape building blocks by stable lateral interactions between 12 subunits. (Reprint from Rao *et al*., 2018, ACS Appl. Mater. Interfaces *10*, 25135–25145.)

### VP1054

VP1054 (Ac54) is a capsid protein encoded by a baculovirus core gene that functions in nucleocapsid assembly. A ts mutant of this protein produced spherical electron-dense structures in the VS in place of nucleocapsids at the non-permissive temperature (Olszewski and Miller, 1997). When *ac54* was knocked out, abnormal elongated empty capsids formed by VP39 were found at the RZ region (although no capsid or VP39 was found at VS), suggesting that VP1054 is involved in the localization of VP39 to VS (Guan *et al.*, 2016). Immunoelectron microscopy showed that VP1054 is critical for the transport of capsid proteins, including VP39, 38K, C42, and P78/83 to the VS for nucleocapsid assembly (Guan *et al.*, 2016).

### Ac53

Ac53 is conserved in all sequenced baculoviruses. Knockout of *ac53* resulted in abnormal electron-lucent tubular structures in both the VS and RZ regions, and BV and ODV production were abolished (Liu *et al.*, 2008). Ac53 was also found to be associated with BVs of AcMNPV (Liu *et al.*, 2008). Although Ac53 is predicted to contain a conserved U-box/RING-like domain of E3 ubiquitin ligases (Garavaglia *et al.*, 2012), the exact role of Ac53 in nucleocapsid assembly requires further investigation.

## BV budding and ODV morphogenesis

Assembled nucleocapsids either egress from nuclei and bud from plasma membrane to form BVs in the early stages of infection or remain within infected nuclei to form ODVs, which are further embedded into OBs in the late stages of infection. It has been suggested that the majority of synthesized viral DNA remains in the nucleus to form ODVs, with only a small portion used to generate BVs. The mechanism of how baculovirus regulates the production of BV and ODV is still unknown. Viral encoded FP25K plays an important role in the switch from BV to ODV production through its effect on the nuclear trafficking of occlusion-associated proteins (Rosas-Acosta *et al.*, 2001; Garretson *et al.*, 2016). Ubiquitination of a nucleocapsid protein Ac66 by virus encoded ubiquitin (v-Ubi) and potential E3 ligase (Ac141, Exon 0) may serve as a signal to determine if a nucleocapsid is destined to form BV or ODV (Biswas *et al.*, 2018; Blissard and Theilmann, 2018).

### BV budding

BV budding is relatively poorly understood. During BV budding, the pre-existing GP64 proteins on the plasma membrane drive nucleocapsid budding. Deletion of *gp64* resulted in an approximately 98% reduction in progeny BV production (Oomens and Blissard, 1999). Host cellular vesicular biogenesis, fusion, and trafficking machineries, including the endosomal sorting complex required for transport (ESCRT) and the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE), are involved in the entry and nuclear egress of BVs (Li and Blissard, 2012; Guo *et al.*, 2017; Yue *et al.*, 2018). Additional work is needed to elucidate the details of the intracellular vesicle transport process of baculoviruses. Data from fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM) showed that VP39 interacts directly with kinesin-1, suggesting that microtubules are involved in anterograde trafficking (towards the plasma membrane) of baculoviruses (Danquah *et al.*, 2012). As previously mentioned, F-actin is a key driver of the nuclear egress of newly assembled nucleocapsids and further BV budding at the plasma membrane (see 'Anterograde transport of nucleocapsid').

### ODV morphogenesis

In the late stage of infection, nucleocapsids are transported to the RZ, a region between the margins of nuclei and the VS, where they acquire envelopes from virus-induced intranuclear membrane vesicles (MVs) to form ODVs. Early electron microscopy studies showed the formation of MVs within the RZ and the association of nucleocapsids with these vesicles via their apical cap ends (Kawamoto *et al.*, 1977; Fraser, 1986). The inner nuclear membranes (INMs) of infected cells bleb, fold in and vesiculate; MVs are derived from the budding of INMs into the nucleoplasm (Tanada and Hess, 1976; Shi *et al.*, 2015). A model for the formation of MVs has been proposed, which includes: (1) disruption of the nuclear lamina underlying the nucleoplasmic face; (2) membrane curvature at the budding sites; and (3) membrane scission at the bud neck to form MVs (Shi *et al.*, 2018). Several

viral genes have been reported to be required for ODV envelopment; however, only *ac75*, *ac76*, and *ac93* have been shown to be required for intranuclear microvesicle formation (Hu *et al.*, 2010; Yuan *et al.*, 2011; Shi *et al.*, 2018). These proteins may participate in the regional disruption of the nuclear lamina or modify the nuclear membrane to promote bending or membrane scission. The morphogenesis of these intranuclear MVs has yet to be demonstrated.

Recently, electron tomography (ET) revealed the dynamic process of microvesicle formation and ODV envelopment in AcMNPV (Shi *et al.*, 2015). The ODV envelopment process can be divided into five steps: (1) the attachment of nucleocapsids to large globular intranuclear vesicles in a vertical fashion; (2) the deformation and rupture of nucleocapsid-associated vesicles; (3) formation of arch-shaped membranes, which grow in size by fusion with small microvesicles; (4) gradual envelopment of the attached nucleocapsids to form immature ODVs containing multiple bundles of nucleocapsids; and (5) invagination of the immature ODV membrane and separation of the bundles of nucleocapsids into individual ODVs, each with a single bundle of parallel nucleocapsids. Whereas it had been generally believed that MVs were derived from the INMs, in this study, the authors found that both the inner and the outer nuclear membranes directly served as the source of the ODV envelopes.

In the final stage, mature ODVs are embedded into OBs. Polymerization of polyhedrin protein appears to follow formation of ODVs in a fairly rapid process (Fraser, 1986; Sajjan and Hinchigeri, 2016). The crystal structure of baculovirus OBs revealed polyhedrin proteins folded into a trimer, with the trimeric interface forming predominantly hydrophobic surfaces. Four identical trimers assemble into a tetrahedral cluster, stabilized mainly by an intermolecular disulfide bond and N-terminal domains. The tetrahedral clusters are further tightly packed in OBs and form unit cells interlocked by the C-terminal arms (Coulibaly *et al.*, 2009). The envelope of mature OBs is composed of polyhedron envelope protein (PEP) and carbohydrate. Deletion of *pep* resulted in OBs with rough pitted surfaces, with many cavities owing to the dislodgment of ODVs (Gross *et al.*, 1994). A recent study employing AFM revealed a multilayered structure for PEP, suggesting that it confers OB stability from environmental adversities (Sajjan and Hinchigeri, 2016).

## Regulation of host immunity, behaviour and physiology

As a result of millions of years of co-evolution with their hosts, baculoviruses have developed different strategies to suppress host defence systems, manipulate host behaviour (induce 'tree-top disease' or 'Wipfelkrankheit') and modulate host physiology (prevent host moulting and induce liquefaction of larval body) for optimal virus replication and dissemination.

### Suppression of host anti-viral immune responses

Insects possess powerful innate immune systems to combat virus infection (Sabin *et al.*, 2010). To date, three innate immune responses, RNA interference (RNAi), apoptosis and melanization, have been implicated as host defences against baculoviruses. To successfully infect their hosts, baculoviruses have evolved multiple strategies to overcome these defence systems.

#### Apoptosis

Apoptosis is programmed cell death executed by a cascade of caspase activation. This process is known to be negatively regulated by cellular inhibitors of apoptosis proteins (IAPs). During baculovirus infection, viral DNA replication triggers host DNA damage responses, which leads to the depletion of host cellular IAPs and the activation of apoptosis (Vandergaast *et al.*, 2011; Mitchell and Friesen, 2012). Baculoviruses encode several types of apoptosis suppressors. P35 is the first identified apoptosis inhibitor (Clem *et al.*, 1991), and was found to abolish a wide range of effector caspases. A divergent homologue of P35, termed P49, can inhibit both initiator and effector caspases. Another baculovirus apoptosis suppressor, Apsup, inhibits initiator caspase (Yamada *et al.*, 2013). Individual baculovirus genomes generally contain only one of these three classes of apoptosis suppressors. Apart from these, most baculoviruses also encode cellular IAP homologues. In fact, IAPs were initially identified in baculoviruses and have since been found in cells (Crook *et al.*,

1993). They are phylogenetically classified into five clades (*iap*1–5). Among these, IAP3 exhibits the broadest anti-apoptotic activity (Ikeda *et al.*, 2013) and likely acts by stabilizing cellular IAPs for their proper functions as evidence by the study in *Orgyia pseudotsugata multiple NPV* (Byers *et al.*, 2016). Not all IAPs block apoptosis; some of them can even have the opposite effect under certain conditions (Ikeda *et al.*, 2011).

### RNAi

RNAi is a critical anti-viral immune response for insects. High throughput sequencing has identified a large number of viral short interfering RNAs (vsRNA) in baculovirus infected cells. Knock-down of *Dicer-2*, an enzyme required for siRNA biogenesis, significantly enhanced viral DNA replication, suggesting that RNAi acts as an important host defence mechanism against baculovirus infection (Jayachandran *et al.*, 2012). To inhibit the defence mechanism, AcMNPV exploits viral protein P35 as a broadly active viral suppressor of RNAi (VSR). The function of P35 in suppression of RNAi is downstream in the RNAi pathway and independent of the P35 anti-apoptotic activity, although the details of its mechanism remains largely unknown (Mehrabadi *et al.*, 2015).

### Melanization

Melanization is a unique and conserved innate defence mechanism in invertebrates. It consists of a cascade of serine proteases that convert prophenoloxidase (PPO) zymogens into the active phenoloxidase (PO) form, which in turn catalyses the formation of melanin to encapsulate and kill invading pathogens. Both *in vitro* and *in vivo* evidence suggest that melanization plays a crucial role in the protection of insects against baculovirus infection (Washburn *et al.*, 1996; Yuan *et al.*, 2017). To counteract this host response, HearNPV appears to have developed two mechanisms: (1) global shutdown of the expression of serine proteases in the melanization cascade; (2) up-regulation of two specific negative regulators (serine protease inhibitors, serpins) (Yuan *et al.*, 2017). Interestingly, certain baculoviruses also encode a *serpin* orthologue and a *conotoxin*-like gene to antagonize host melanization (Cao *et al.*, 2012; Ardisson-Araujo *et al.*, 2015).

## Manipulation of host behaviour

Baculovirus infection induces enhanced locomotor activity (ELA) of host insects, including 'hyperactivity' and 'tree-top disease'. These behaviour changes are thought to increase the dispersal of OBs, since insects climbing to the top of the tree enhance visibility to predators. Two viral proteins have been implicated in inducing ELA in infected hosts.

### PTP

PTP, a virus-encoded protein tyrosine phosphatase, was identified as responsible for 'wandering-like' ELA via screening of a gene-knockout library of BmNPV (Kamita *et al.*, 2005). PTP-induced ELA is likely to be conserved in a subset of baculoviruses (van Houte *et al.*, 2012; Katsuma *et al.*, 2012). However, the phosphatase activity of PTP may not be conservatively required for ELA induction, since there are contradictory reports in different virus–host systems. Homologues of *ptp* only occur in Group I alphabaculoviruses; this gene could also be captured from insect hosts. In fact, a host PTP homologue was found to partially replace the function of BmNPV PTP (Kamita *et al.*, 2005).

### EGT

EGT, a virus encoded ecdysteroid (UDP)-glucosyltransferase, may be involved in tree-top disease. The deletion of the *egt* gene in *Lymantria dispar multiple nucleopolyhedrovirus* (LdMNPV) and *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV) led to the death of host larvae at lower positions compared to the wild-type viruses (Hoover *et al.*, 2011; Han *et al.*, 2015). However, this does not appear to be the case in *T. ni* and *S. exigua* larvae infected by an *egt*-deleted AcMNPV (Ros *et al.*, 2015). It has recently been suggested that EGT-induced tree-top disease could be an indirect effect of moulting (Zhang, S. *et al.*, 2018).

## Regulation of host physiology

Larvae stop feeding and become quiescent during moulting, which reduces baculovirus proliferation. EGT can inactivate host moulting hormones by catalysing the conjugation of glucose and galactose to ecdysteroids, which interferes with normal insect moulting (O'Reilly and Miller, 1989). This leads to prolonged insect feeding times and increased yield of viral progeny. Accordingly, deletion of *egt*

improves the efficacy of baculoviral pesticides, significantly reducing foliage consumption and increasing speed of action as compared to wild type viruses (O'Reilly and Miller, 1991). This gene likely has been captured by baculoviruses from their insect hosts for optimal virus proliferation.

The rigid exoskeleton of chitin poses a barrier for the release of virus progeny. At the late stage of infection, larval tissues undergo liquefaction, which is executed by two virus-encoded enzymes, chitinase and cathepsin (Ohkawa *et al*., 1994; Hawtin *et al*., 1997). Insects infected with recombinant viruses lacking either *chitinase* or *cathepsin* genes remain intact after death (Hawtin *et al*., 1997). Chitinase and cathepsin are stored in the endoplasmic reticulum (ER) to prevent pre-mature degradation of host tissues and are liberated upon host death. Thus, OBs are released from the liquefied larval corpse to contaminate surrounding foliage or soils, for ingestion by new host insects to begin another infection cycle.

## Synthetic biology in baculoviruses

Viruses are ideal models for synthetic biology research because of their small genome size. Viruses are likely the most readily reconstituted form of life. A small RNA virus (poliovirus) was the first organism to be synthesized, in 2002 (Cello *et al*., 2002). The unique life cycle of baculovirus with its two phenotypes (BV and ODV) provides an excellent model system for synthetic biology. For example, some of the ODV-specific genes that are non-essential for BV production can be deleted from the virus genome for both fundamental and applied research.

Shang *et al*. (2017) reported the first synthesis of the prototypical baculovirus, AcMNPV, by using a combination of overlap extension PCR and transformation-associated recombination (TAR) in yeast. The synthetic genome, designated AcMNPV-WIV-Syn1, is approximately 145 kb, encompassing almost the complete genome of AcMNPV. A cassette of about 11.5 kb including bacterial and yeast replication elements and an *egfp* gene in the *hr4a* locus were added to the synthetic genome. Further transfection of AcMNPV-WIV-Syn1 DNA into insect cells produced infectious virus that retained similar biological properties (infectivity and virion morphology) to the native virus, a major step towards genome-wide editing of baculovirus.

This new synthetic platform offers the opportunity to manipulate any or multiple loci simultaneously, thus providing a powerful tool for further investigation of the basic biology as well as engineering of baculoviruses. Potential future research directions include (1) construction of a mini-genome of baculovirus to understand the elements required for a minimal functional baculovirus; (2) deletion of irrelevant genes for BV production from the baculovirus genome to engineer a vector with larger insertion capacities for gene expression and delivery; (3) adding entire mammalian protein modification pathways into the baculovirus genome to facilitate better production of foreign proteins and vaccines; and (4) substitution of a whole set of heterologous PIFs for studying the oral infection mechanism and expansion of the host ranges of baculoviral pesticides.

To date, approximately 45% of the AcMNPV genome has been classified as inessential for BV production. This might be verified by the 'Design-Build-Test Cycle' strategy used by the laboratory of Craig Venter for synthesis of a minimal bacterial genome (Hutchison *et al*., 2016). Both 'top-down' and 'bottom-up' synthetic biology approaches might be used to synthesize such a mini-genome for AcMNPV (Wang and Maranas, 2018) (Fig. 9.7). The resulting minimal functional baculovirus would provide pivotal fundamental information for understanding baculovirus functional genomics and evolution, as well as being beneficial for designing novel gene expression and delivery vectors with enhanced stability and greater packaging capacities.

## Conclusions and future perspectives

Over the past few decades, remarkable progress has been made towards understanding baculovirus genome organization, structures of both virion phenotypes, functional genomics, regulation of viral gene expression, and baculovirus–host interaction. However, numerous fundamental questions remain. The structure and function of individual PIFs and of the PIF complex have yet to be determined. The cellular receptors for ODV and BV entry are likewise still mysteries. Viral proteins and host factors for mediating the nuclear import of nucleocapsids

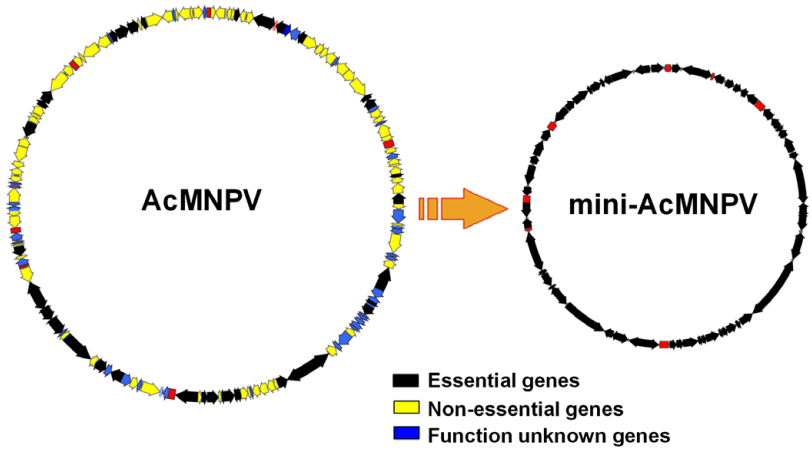


**Figure 9.7** Schematic representation of an AcMNPV mini-genome. Approximately 45% of the AcMNPV genome is estimated to be non-essential (yellow) for BV production. After validating the impacts of unknown genes on BV production, a mini-genome of AcMNPV can be designed and constructed by removal of the non-essential genes using synthetic biology approaches.

are completely unknown. In addition, knowledge of how baculoviruses modulate the host immune system and manipulate host physiology/behaviour for better virus replication and dissemination is limited. With the expanded potential of technologies such as Cryo-EM, CRISPR/Cas9, and synthetic biology platforms, these questions will likely be addressed in the near future. The answers will shed light on the baculovirus infection mechanisms, providing useful information and important strategies for further engineering of baculoviruses into more efficient protein expression vectors and viral pesticides.

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