Characterization of a nucleopolyhedrovirus with a deletion of the baculovirus core gene Bm67

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Open reading frame (ORF) 67 (Bm67) of the Bombyx mori nucleopolyhedrovirus (BmNPV) is a highly conserved gene that is found in all completely sequenced baculoviruses; its function is unknown. In the present study, a Bm67-knockout virus was generated for studying the role of Bm67 in the BmNPV infection cycle. Furthermore, a Bm67-repair bacmid was constructed by transposing the Bm67 native promoter-promoted Bm67 ORF into the polyhedrin locus of the Bm67-knockout bacmid. After these recombinant bacmids were transfected into BmN cells, the Bm67-knockout bacmid caused defects in the production of infectious budded viruses. However, the Bm67-repair bacmid could rescue the defect, and budded virus titres reached wild-type levels. Quantitative real-time PCR analysis indicated that Bm67 is required for normal levels of DNA synthesis or for the stability of nascent viral DNA at the early stage. Electron microscopic analysis revealed that the formation of normal-appearing nucleocapsids is reduced in Bm67-knockout bacmid-transfected cells, and nucleocapsids are rarely found in the cytoplasm. The presence of ‘enveloped’ nucleocapsids at the nucleoplasm bilayer indicated that they are enveloped abnormally. These results indicated that Bm67 is required for the production of infectious budded viruses and for assembly of envelope and nucleocapsids.

INTRODUCTION

Baculoviruses are characterized by a large, double-stranded DNA genome, ranging in size from 80 to 180 kb, that is packaged within a rod-shaped capsid and enclosed by a lipid envelope (Herniou et al., 2003). In a single infection cycle, two morphologically distinct virion phenotype are produced: budded virions (BV) and occlusion-derived virions (ODV). Although the nucleocapsids of BVs and ODVs are structurally similar, the origin and composition of their envelope proteins differ. The BVs acquire an envelope by budding from the host-cell plasma membrane, whereas the envelope of ODVs is acquired from within the nucleus of the host cell. It is likely that these two morphologically different, but genetically identical, viral forms are a reflection of their different roles during infection (Braunagel & Summers, 1994).

The infection cycle of the baculovirus can be subdivided into three major phases of viral transcription: early, late and very late. In the early stage, replication of the virus genome occurs in specific nuclear regions; later, replication proceeds in almost the entire nucleus and is associated with virogenic stroma in which the newly replicated viral DNA is condensed and packaged to form nucleocapsids. Nucleocapsids can bud through the nucleoplasm and enter the cytoplasm. When enveloped nucleocapsids bud through the modified plasma membrane, they acquire the most important envelope protein, GP64, which is essential for forming a mature BV. GP64 is an essential virion structural protein that is required for propagation of the BV from cell to cell and for systemic infection of the host insect (Monsma et al., 1996). Later in infection, nucleocapsids remain within the nucleus, become bundled together and are enveloped by a membrane elaborated within the nucleus to form a mature ODV. In the very late stage, polyhedrin (ph), the major matrix protein for mature occluded viruses, is hyperexpressed. The resulting ODVs are embedded and packaged within occlusion bodies, also known as polyhedra.

Although the average baculovirus genome encodes 90–180 open reading frames (ORFs), more than 800 different orthologous gene groups were identified in 29 sequenced baculovirus genomes (Jehle et al., 2006). Thus, only a small proportion of the viral genome encodes genes that are common to all baculoviruses. Comparative analysis of the completely sequenced 29 baculovirus genomes shows that there are 29 identified genes in common; these are grouped as the baculovirus core set genes (Herniou et al., 2003). Bombyx mori nucleopolyhedrovirus (BmNPV) is one of the main baculoviruses that has been studied extensively. The BmNPV (T3 strain) genome has been completely sequenced; it is 128 413 nt in length and contains 136 ORFs that encode predicted proteins of >60 aa (Gomi et al., 1999). Although the transcription pattern and
functions of the majority of BmNPV ORFs, including their homologues, have been clear, there are still several genes whose features and functions remain unknown, including BmNPV ORF67 (Bm67). Bm67 is 705 nt in length and is located at nt 61190–61892 in the BmNPV (T3 strain) genome. It is one of the 29 baculovirus core set genes.

To determine the role of Bm67 in the virus life cycle, a BmNPV bacmid was used to generate a Bm67-knockout mutant by homologous recombination in *Escherichia coli*. Our data indicated that Bm67 is required for normal levels of DNA synthesis or for the stability of nascent viral DNA at the early stage. Further, it is essential for BV production; electron microscopic analysis demonstrated that Bm67 is required for normal envelope assembly.

**METHODS**

**Cells and viruses.** BmNPV (ZJ strain) and BmNPV bacmid virus were propagated in BmN (BmN-4) cells, maintained at 27 °C in TC-100 medium (Sigma) supplemented with 10% (v/v) fetal bovine serum.

**Bacterial strains, bacmid DNA and plasmids.** *E. coli* strain BW25113 (pKD46) and plasmid pKD46, containing the phage λ Red system under the control of the arabinose promoter, were kindly provided by Dr Mary Berlyn (Yale University, CT, USA). *E. coli* strain DH10Bac (Invitrogen) was used to isolate the helper plasmid (pMON7124), which encodes a transposase. *E. coli* strain BmDH10B, containing BmNPV bacmid (BmBac) DNA, was provided by Dr Enoch Y. Park (Shizuoka University, Japan).

**Generation of the Bm67-knockout bacmid.** The Bm67-knockout Bacmid was generated by using the λ Red recombination system as described previously (Lin & Blissard, 2002a; Lung et al., 2003; Vanarsdall et al., 2004; Wu et al., 2006). First, we generated a transfer vector in which the CAT cassette was inserted into the Bm67 gene to replace a 132 bp region. The 5′ flanking region of the Bm67 ORF (BmNPV nt 61656–62093) was amplified by PCR using the BmNPV bacmid and the primer pair FF (5′-AGATCTGTTCGACGGACAACTATAG-3′) and FR (5′-GTTTTCCCAGTCACGAC-3′) (BamHI and PsI sites are underlined, respectively). The PCR product was digested with BamHI and PsI and then cloned into the vector pETBlue-2 (Novagen) to generate a recombinant plasmid named pET-2-De67F. The 3′ region of the Bm67 ORF (BmNPV nt 61010–61523) was PCR-amplified with the primer pair DF (5′-AAGCTTATATACACCGGACGCC-3′) and DR (5′-CTCGAGATTACGCAGTTTGTG-3′) (BamHI and XhoI sites are underlined, respectively). The PCR product was digested with BamHI and XhoI and cloned into plasmid pET-2-De67F to generate plasmid pET-2-De67F-D. The CAT cassette was PCR-amplified from the pRADZ3 plasmid by using the primer pair CATF (5′-AGACTTCTGAGTTATATATGTCGAC-3′) and CATR (5′-TGAGTTATATGTCGAC-3′) (BamHI and PsI sites are underlined, respectively). The PCR product was cloned into the pMD18-T vector (TaKaRa) and then digested with PsI and KpnI and ligated into the plasmid pET-2-De67F-D to generate the transfer vector pET-2-De67F-CAT-D. The recombinant vector was verified by DNA sequencing. Subsequently, the transfer vector was digested with BamHI and XhoI to produce the linear DNA fragment De67F-CAT-D. The linear fragment (0.1 μg) was then transformed into *E. coli* pKD46/BmBacmid/BW25113 electrocompeotent cells. Transformed cells were incubated at 37 °C for 4 h in 1 ml SOC medium (Sigma), and colonies resistant to chloramphenicol and kanamycin on Luria–Bertani (LB) medium plates were selected and confirmed by PCR analysis.

**Construction of knockout, repair and wild-type (wt) bacmids containing polyhedrin and egfp.** To determine the effect of Bm67 deletion on occlusion morphogenesis and to facilitate the examination of virus infection, the polyhedrin (ph) and enhanced green fluorescent protein (egfp) genes were inserted into the polyhedrin locus by using a site-specific transposon (Luckow et al., 1993; Wu et al., 2006).

The ph gene-coding sequence plus promoter region and poly(A) region were amplified from BmNPV DNA by PCR using the primer pair proPH (5′-GAATTCCTGAGTTATATATGTCGAC-3′) and polyPH (5′-CTCGAGATTACGCAGTTTGTG-3′) (EcoRI and SnaBI sites are underlined, respectively). The ph promoter from pFastBac1 (Invitrogen) was removed, and the ph gene and the AcNPV cl promoter-promoted egfp gene were inserted to generate the donor plasmid pFB1-PH-IG. A 1229 bp repair fragment containing the Bm67 gene promoter, ORF and 3′ untranslated region was amplified by PCR using the BmNPV bacmid as the template and the primer pair ReBm67F (5′-GAATTCCTGAGTTATATATGTCGAC-3′) and ReBm67R (5′-CTCGAGATTACGCAGTTTGTG-3′) (EcoRI and PsI sites are underlined, respectively). The PCR product was cloned into pFB1-PH-IG to generate the repair transfer plasmid pFB1-REP-PH-IG.

The Bm67-knockout bacmid and helper plasmid pMON7124 (Luckow et al., 1993) were electroporated into *E. coli* DH10B cells. The electrocompetent DH10B cells were transformed with the donor plasmids pFB1-PH-IG or pFB1-REP-PH-IG to generate the Bm67-knockout bacmid and the Bm67-repair bacmid, respectively. BmDH10Bac cells, which contain a BmNPV bacmid, were transformed with pFB1-PH-IG to generate the Bm67-deletion control virus Bm67-ko (Fig. 1b).

Each correct recombinant bacmid was isolated and electroporated into *E. coli* DH10B cells and then selected on kanamycin/LB medium plates; colonies were screened for tetracycline sensitivity and DNA was analysed on agarose gels to ensure the absence of helper plasmids. Helper-free bacmid DNA was extracted and purified from each resultant *E. coli* strain by using a Large-Construct kit (Qiagen) according to the manufacturer’s instructions.

**PCR confirmation of recombinant bacmids.** Four PCR primer pairs were used to confirm the Bm67 locus in the selected bacmids. The primers Bm62283 (5′-GTCACTTATATACACCGGACGCC-3′) and Bm59987 (5′-CTCGAGTTATATATGTCGAC-3′) were used to detect the correct insertion of the CAT cassette. Primers CATF and CATR were used to confirm the correct insertion of the CAT cassette. Primer pairs Bm62283/CATR and CATF/Bm59987 were used to examine the recombination junctions of the upstream and downstream flanking regions, respectively.

PCR analysis was also used to confirm the reinsertion of ph, egfp and the repair gene into the polyhedrin locus after site-specific transposon. The primer pair M13F (5′-GTTTTCCCAGTCACGAC-3′) and M13R (5′-CTCGAGATTACGCAGTTTGTG-3′) was used to verify the presence of the transposon. Primer pairs M13F/ReBm67R and ReBm67F/M13R were used to verify the correct insertion of the repair gene Bm67.

**Analysis of viral growth curves.** BMN cells (2 × 10⁵) were transfected with 2.0 μg DNA extracted from each bacmid by using a TransFast liposome (Promega) or infected with BV at an m.o.i. of 5. The cells were washed three times with TC-100 medium after incubation for 8 h after transfection or 1 h after infection; the cells were then supplied with 2 ml fresh TC-100 medium supplemented with 10% fetal bovine serum. Virus supernatants were collected at selected time points. BV titres were determined by a TCID₅₀ endpoint dilution assay using BMN cells (O’Reilly et al., 1994).
Transfection–infection assay. A transfection–infection assay was performed to examine the viral replication of Bm67-knockout bacmids in BmN cells (Lin & Blissard, 2002a, b). BmN cells were transfected with vBm, vBmKO or vBmKO-R. At 6 days post-transfection (p.t.), supernatants were removed from the transfected cells and added to a second group of freshly plated BmN cells. These cells were then incubated for up to 12 days and examined daily by fluorescence microscopy.

Quantitative real-time PCR (Q-PCR) DNA replication assay. To detect viral DNA replication, a Q-PCR assay was performed as described by Vanarsdall et al. (2005, 2006). The primers Bm60352F (5'-CGTAGTGGTAGTAATCGCCGC-3') and Bm60452R (5'-AGTCGAGTCGCGTCGCTTT-3') were used to amplify a 101 bp region within the gp41 ORF of BmNPV. There are three DpnI restriction sites in this region; hence, DpnI can be used to digest the input bacmid DNA. BmN cells were transfected with vBm or vBmKO and harvested at different time points. Total DNA was extracted and digested with DpnI (Fermentas). Digested DNA (5.0 ng) was mixed with SYBR Premix Ex Taq (TaKaRa) according to the manufacturer’s instructions. Q-PCR was performed by using the iCycler iQ Multicolor Real-Time PCR Detection system (Bio-Rad) under the following conditions: 95°C for 30 s; 45 cycles of 95°C for 5 s and 60°C for 20 s, with a 500 mM concentration of each primer.

Electron microscopy. BmN cells (2 x 10^6) were transfected with 5.0 μg vBm or vBmKO and the cells were harvested for transmission electron microscopy at 3 days p.t. Mock-transfected cells were treated similarly, but without the addition of virus DNA. To observe the change of transfected cells at the extremely late stage, the cells transfected with Bm67-knockout virus were harvested at 12 days p.t. for electron microscopy, and the cell-culture medium of the transfected cells was replaced with fresh TC-100 medium at 6 days p.t. The harvested cells were fixed in their transfection dishes with 2.5 % glutaraldehyde for 1 h, then dislodged and centrifuged at 1000 g for 5 min. Cell pellets were washed three times in 0.1 M PBS, embedded in 2 % agarose and fixed again in 2.5 % glutaraldehyde overnight. They were then washed and post-fixed in 1 % osmium tetroxide for 1 h. The samples were dehydrated in a standard ethanol/acetone series, infiltrated and embedded in Spurr medium, and then superthin sections were cut. The sections were stained with 5 % uranyl acetate followed by Reynolds’ lead citrate solution and viewed under a JEM-1230 transmission electron microscope.

RESULTS

Construction of Bm67-disruption and -repair bacmids

First, we constructed a transfer vector (pET-2-De67F-CAT-D) that contains a 438 bp 5’ flanking region and a 514 bp 3’ flanking region of Bm67 and a 974 bp CAT cassette in the middle (Fig. 1a). Fragments of the 5’ and 3’ ends of the Bm67 ORF region (237 and 514 bp, respectively) were retained, to prevent the deletion of Bm67 in the BmNPV bacmid from influencing the transcription of the neighbouring ORFs, namely Bm68 and gp41. The linear DNA fragment De67F-CAT-D was transformed into electrocompetent cells of E. coli strain pKD46/BmBacmid/BW25113, which express the λ phage recombination genes. The resulting recombinant bacmid was

![Fig. 1. Construction and analysis of Bm67-knockout and -repair and wt BmNPV bacmids.](image-url)
confirmed by a combination of PCR analyses using four primer pairs (Fig. 1a).

To confirm the phenotype resulting from the Bm67 knockout and to facilitate the examination of virus infection, three recombinant bacmids were generated: the Bm67-knockout bacmid vBmKO, the repair bacmid vBmKO-R (Fig. 1a) and the positive-control wt bacmid vBm (Fig. 1b).

All constructs were confirmed by PCR analysis with diagnostic primers (Fig. 1a); the results are shown in Fig. 1(c). PCR with the primer pair Bm62283/Bm59987 produced 3271 bp fragments for vBmKO and vBmKO-R, but 2297 bp fragments for vBm. PCR with the primer pair CATF/CATR yielded a single 974 bp fragment for both vBmKO and vBmKO-R, but no PCR product for vBm, indicating that the CAT cassette was inserted correctly. The primer pairs Bm62283/CATR and CATF/Bm59987 produced fragments of 1602 and 2509 bp, respectively, for both vBmKO and vBmKO-R, but no PCR product for vBm, which indicated that recombination occurred at the correct junctions of the upstream and downstream flanking regions. Tn7-mediated transposition was also confirmed by PCR with specific primers (Fig. 1d). The results indicated that the ph, egfp and wt Bm67 genes had been transposed correctly into the polyhedrin locus.

Analysis of viral replication in BmN cells

To determine the effect of Bm67 disruption on virus replication, BmN cells were transfected with vBm, vBmKO or vBmKO-R, and the cells were monitored by fluorescence microscopy. A similar number of vBm-, vBmKO- or vBmKO-R-transfected cells were observed to emit fluorescence at 48 h p.t. At 120 h p.t., vBmKO-transfected cells showed almost no increase in the number of infected cells, but fluorescence was observed in almost all vBm- or vBmKO-R-transfected cells (Fig. 2a). This indicated that Bm67-knockout bacmid-transfected cells could not generate infectious BVs from the initial infection, but reinsertion of Bm67 into the polyhedrin locus rescued cells from this defect. To confirm the observations, an initial transfection–infection assay was performed as described by Lin & Blissard (2002a, b). The results revealed that no fluorescence was detected in BmN cells incubated with the supernatant obtained from vBmKO-transfected cells; however, cells incubated with the supernatants of vBm- or vBmKO-R-transfected cells demonstrated the characteristics of virus infection and, further, fluorescence was observed in almost all cells at 96 h p.t. (Fig. 2b). This indicated that Bm67 is essential for viral infection in BmN cells.

To assess further the effect of Bm67 knockout on virus production, a one-step virus growth-curve analysis was performed. Virus production was not detected from the vBmKO-transfected cells, but the vBm- or vBmKO-R-transfected cells revealed a steady increase in virus production (Fig. 2c, transfection). A virus infection growth curve revealed that reinserting Bm67 into the polyhedrin locus of the Bm67-knockout bacmid could rescue the defect in BV production of the Bm67-knockout virus completely (Fig. 2c, infection). Thus, the defect in BV production by the Bm67 knockout virus was due to the
disruption of Bm67; hence, we can conclude that Bm67 is essential for virus propagation in cultured BmN cells.

**Bm67 deletion reduced viral DNA replication at the early stage**

To quantify viral DNA replication in BmN cells, a six-step standard calibration curve was generated by using purified vBm bacmid DNA diluted serially from 10 to 0.00001 ng (Fig. 3a). The dissociation curve showed that the Q-PCR-detected fluorescence was due to the specific amplification of the homologous DNA sequence (data not shown).

To detect viral DNA replication in BmN cells, identical numbers of vBmKO- and vBm-transfected BmN cells were collected at designated time points, and total DNA was obtained by phenol extraction followed by ethanol precipitation. The extracted DNA was digested with DpnI and used for real-time PCR. The results indicate that DNA synthesis by vBmKO is reduced at the early stage (Fig. 3b). At 0 h p.t., similar amounts of viral DNA were obtained from cells transfected with vBmKO or vBm; this indicated that equal amounts of DNA were transfected. The quantity of DNA in vBmKO was lower than that in vBm at 12 and 24 h p.t. As BVs are produced by wt viruses at approximately 12 h post-infection (p.i.) and the production increases exponentially until approximately 20 h p.i. (Knudson & Harrap, 1975), the BVs can initiate secondary infection leading to an increase in the number of cells containing viral DNA after 24 h p.i. In contrast, vBmKO could only replicate in cells that were initially transfected, indicating that Bm67 is required for normal levels of DNA synthesis or for the stability of nascent viral DNA at the early stage. An analysis of viral DNA replication from 48 to 96 h p.t. revealed a substantial and steady increase in the amount of viral DNA generated by the wt bacmids. Although the quantity of viral DNA generated by the Bm67-knockout bacmids also increased, the increase was much lower, as seen by comparing the quantity of viral DNA generated by the vBm. An increase in viral DNA during days 4–12 p.t. was detected in the cells transfected with vBmKO, indicating continuous viral DNA synthesis.

**Transmission electron microscopic analysis of wt and Bm67-knockout bacmid-transfected cells**

To analyse further whether the deletion of Bm67 had any effect on virus morphogenesis, electron microscopic analysis was performed using thin sections obtained from the wt and Bm67-knockout virus-transfected cells (Fig. 4). At 3 days p.t., cells transfected with the Bm67 wt virus revealed the following: symptoms typical of nucleopolyhedrovirus (NPV) infection and enveloped virions that were occluded or being occluded into polyhedra (Fig. 4a). Cells transfected with vBmKO also revealed cytopathological changes in the nucleus at 3 days p.t.; many loose, granular materials were seen dispersed throughout the nucleoplasm, and clumps of heterochromatin were displaced peripherally by the forming stroma. However, no typical polyhedra were observed (Fig. 4b). Nucleocapsids were observed within cells transfected with either vBm or vBmKO, but the number of nucleocapsids in cells transfected with vBmKO was much lower than that in cells transfected with vBm (Fig. 4c). At 6 days p.t., cells transfected with vBm underwent apoptosis. In contrast, cells transfected with vBmKO remained intact; however, the number of nucleocapsids increased to many more than those present at 3 days p.t. To observe whether the cells transfected with
vBmKO underwent any changes at the extremely late stage, they were maintained for 12 days p.t. until some of the cells underwent apoptosis. At 12 days p.t., most of the cells transfected with vBmKO also appeared intact; virogenic stroma were observed within the nucleus at the putative sites of nucleocapsid assembly (Fig. 4e), the intrastromal spaces were rich with developing nucleocapsids, and mature nucleocapsids were usually observed at the electron-dense edges of the stroma (Fig. 4f), very few of which were found in the cytoplasm (Fig. 4d). Regularly arranged bundles of nucleocapsids were observed; however, we rarely detected an empty envelope or enveloped virions in the nucleus (Fig. 4g). It was observed that many nucleocapsids were ‘enveloped’ within the inner nuclear membrane at the nucleoplasm bilayer (Fig. 4h). These observations indicated that the deletion of Bm67 reduced nucleocapsid assembly and resulted in a defect in envelope formation and assembly with nucleocapsids.

DISCUSSION

To examine the role of Bm67 during the infection cycle, a Bm67-knockout bacmid was generated by recombination in E. coli. After transfecting the Bm67-knockout bacmid into BmN cells, the infection was restricted to the cells initially transfected; the Bm67-knockout virus was unable to propagate in the BmN cells, and this was confirmed by an initial transfection–infection assay. The defect in replication could be rescued by reinserting the Bm67 gene into the polyhedrin locus of the same bacmid, thus confirming that the observed phenotype resulted from
the Bm67 knockout and not from a mutation at a second site or by the disruption of regulatory elements located at the Bm67 locus. Therefore, Bm67 is an essential gene for BmNPV virion propagation in BmN cells.

Q-PCR analysis showed that the quantity of DNA in the Bm67-knockout bacmid-transfected cells was less than that of the wt virus at the initial 24 h p.t., suggesting that Bm67 is required for normal levels of DNA synthesis or for the stability of nascent viral DNA at the early stage. Although the mechanism of baculovirus genome replication is unclear, there is evidence that many viral replication factors are involved in the process (Hefferon & Miller, 2002; Mikhailov, 2003; Okano et al., 2006). Replication is associated with a specific structure known as virogenic stroma, in which many replication factors, such as IE1, IE2 and the DNA-binding protein (DBP), accumulate (Mainz et al., 2002; Okano et al., 1999). BmNPV deletion mutants lacking P35, IE2, DBP or LEF-7 showed reduced DNA synthesis, but these proteins were not essential for virus DNA replication (Gomi et al., 1997). The exact role of Bm67 in the virus DNA replication process needs to be clarified further.

A homologue search showed that Bm67 is highly conserved among baculoviruses; this suggests that the homologues may play an identical role in the baculovirus infection cycle. HaSNPV ORF74 (Ha74) is a homologue of Bm67 and demonstrated 51% identity with the predicted protein sequence. To determine whether a homologue of Bm67 from group II NPVs could rescue the defect of Bm67 knockout in group I NPVs, we generated a repair bacmid containing polyhedrin, egfp and the Bm67 promoter-promoted Ha74 ORF. The results showed that Ha74 was unable to rescue the defect in viral replication resulting from the deletion of Bm67 (data not shown).

To determine whether Bm67 and its homologues are species-specific, more gene homologues from other group I and group II NPVs need to be tested. Baculoviruses have been identified in over 800 different insect species thus far. Most baculoviruses have limited host ranges and infect one or a few closely related insect species from a single order; infection may even be restricted to a single insect host (Federici, 1997). Several genes have been shown to be involved in the determination of host specificity, e.g. helicase (Crozier et al., 1994), P35 (Clem & Miller, 1993), HCF-1 (Hefferon, 2003; Lu & Miller, 1996), P74 (Wu et al., 2003) and HRF-1 (Ikeda et al., 2005). The ability to inhibit baculovirus-induced apoptosis or to overcome the premature and general cessation of both viral and host protein synthesis is an important factor in the host-range determination of some baculoviruses (Iwanaga et al., 2004). The complete expression of early and late genes and the synthesis of normal levels of viral DNA also restrict the host range of baculoviruses (Morris & Miller, 1993). As virus replication is reduced in the early stage in Bm67-knockout virus-transfected cells, Bm67 may bind to a special DNA sequence or interact with DNA-binding proteins to stimulate replication; it may also be involved in the expression of host-specificity genes.

Electron microscopy revealed that the Bm67-knockout virus can produce nucleocapsids of normal appearance, but the number of nucleocapsids was much lower than those in the control virus, and these nucleocapsids have defects in envelope assembly. Many genes are considered to be associated with baculovirus assembly; most of them encode structural proteins and are conserved in baculoviruses, e.g. GP64 (Oomens & Blissard, 1999), alkaline nuclease (Okano et al., 2007), GP41 (Olszewski & Miller, 1997b), VP1054 (Olszewski & Miller, 1997a), VLF-1 (Vanarsdall et al., 2006) and 38K (Wu et al., 2006). Combination techniques were used to identify the proteins present within or associated with ODV, but Bm67 was not identified by these techniques (Braunagel et al., 2003). However, Culex nigripalpus NPV ORF106, a homologue of Bm67, is considered to be associated with ODV (Pereira et al., 2007). One high-scoring transmembrane region (aa 194–210) of the predicted Bm67 protein was identified by the TMpred software (http://www.ch.embnet.org/software/TMPRED_form.html). All of these observations suggest that Bm67 may be an envelope protein of BmNPV, possibly explaining the defect in envelope formation by the Bm67-knockout virus.

Bm67 is localized upstream of gp41 and downstream of Bm68 in the BmNPV genome; the homologues of these three genes are found in the same relative position in most sequenced baculovirus genomes. Considering all of the characteristics of Bm67 and its knockout virus, the Bm67 gene appears to have some similarity to its neighbours. The gp41 gene is also conserved among baculoviruses and is known as a late expression gene (Whitford & Faulkner, 1992a). The GP41 protein was present in the nucleus of infected cells from 12 to 96 h p.i., whereas it was present in the cytoplasm from 24 to 96 h p.i. (Pan et al., 2005). Structural localization confirmed that GP41 is associated with the envelope of ODVs (Whitford & Faulkner, 1992b). Nucleocapsid production in the nucleus persisted even with gp41 mutation, and GP41 is required for the nucleocapsid to enter the cytoplasm from the nucleus, which is one of the steps in the pathway of BV synthesis (Olszewski & Miller, 1997b). Bm68 is also demonstrated to be a late gene and it encodes a structural BV protein. Viruses with Bm68 deletion demonstrated reduced budding efficiency and delayed viral DNA accumulation (Iwanaga et al., 2002). The presence of gene clusters may be due to physical constraints that prevented their separation (Herniou et al., 2003); whether such gene clustering has any relevance in the functional process is not clear.

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