BM61 of *Bombyx mori* nucleopolyhedrovirus: Its involvement in the egress of nucleocapsids from the nucleus

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**A B S T R A C T**

All lepidopteran baculovirus genomes sequenced encode a homolog of the *Bombyx mori* nucleopolyhedrovirus *orf61* gene (*Bm61*). To determine the role of *Bm61* in the baculoviral life cycle, we constructed a *Bm61* knockout virus and characterized it in cells. We observed that the *Bm61* deletion bacmid led to a defect in production of infectious budded virus (BV). Quantitative PCR analysis of BV in the media cultureting the transfected cell indicated that BV was not produced due to *Bm61* deletion. Electron microscope analysis showed that in the knockout of *Bm61*, nucleocapsids were not transported from the nucleus to the cytoplasm. From these results we concluded that BM61 is required in the BV pathway for the egress of nucleocapsids from the nucleus to the cytoplasm.

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1. Introduction

The family Baculoviridae comprises a diverse group of arthropod-specific DNA viruses, and is characterized by a circular double-stranded DNA genome, ranging from 80 to 180 kb, packaged within a rod-shaped capsid and enclosed by a lipid envelope [1,2]. The Baculoviridae contains four genera: alphabaculovirus, betabaculovirus, gammabaculovirus, and deltabaculovirus [3]. Alphabaculovirus and betabaculovirus mainly infect invertebrate species belonging to the order Lepidoptera [4].

The BV and occlusion-derived virus (ODV) forms have different functions in the Baculoviridae life cycle. During the early phase of infection, newly replicated viral DNA is condensed and packaged into capsid structures within the nucleus to form nucleocapsids [5]. Then nucleocapsids initially egress from the nucleus, migrate through the cytoplasm, and bud through a modified plasma membrane to acquire an envelope to form BVs [6]. BVs are required for the dissemination of a viral infection throughout the tissues of an infected host. During the late phase of infection, nucleocapsids remain within the nucleus and develop an envelope de novo to form ODVs, which then become incorporated within the matrix of the occlusion body [7], which is required for inter host transmission. Therefore, BV and ODV are genetically identical but differ in their envelope compositions and tissue tropisms, and are produced at different times during infection [8,9].

The *Bm61* of *Bombyx mori* nucleopolyhedrovirus (*BmNPV*) (nt 57499–57898) encodes a putative protein of 133 amino acids with a predicted molecular mass of 15.5 kDa [10]. Based on the comparative analysis of 29 baculoviruses, 62 *orfs* have been identified in common and designated as baculovirus core genes, which are generally essential for viral replication, implying that they play fundamental roles in the baculovirus life cycle [11]. *Bm61* is one of the 62 baculovirus core genes [12]. Homologues of *Bm61* have been identified in genomes of all lepidopteran baculoviruses [13]. Sequence-based queries performed with Inter ProScan program showed that BM61 is a late protein [14], but the detailed function of *Bm61* is still unknown.

We used a *BmNPV* bacmid to generate a *Bm61* deletion by homologous recombination in *Escherichia coli*. Our data with this and other constructs indicate that BM61 is required in the BV pathway for the egress of nucleocapsids from the nucleus to the cytoplasm.

2. Materials and methods

2.1. Cells, virus, bacterial strains, and antibiotics

*BmNPV* (T3 strain) virus was propagated in BmN-4 cells maintained at 27°C in TC-100 insect medium (Gibco, USA)
supplemented with 10% (v/v) fetal bovine serum (Gibco, USA). The E. coli strains BW25113 harboring plasmid pKD46 encoding the λ Red recombination system and BW25114 harboring plasmid pKD3 encoding the chloramphenicol resistance gene (Cm) gene were kindly provided by Mary Berlyn (Yale university). The DH10 harboring BmNPV bacmid and the helper plasmid pMON7124 were kindly provided by Enoch Y. Park (Shizuoka, University).

### 2.2. Knockout and repair bacmid construction

Bacmids containing deletions of Bm61 were made using the λ Red homologous recombination system in E. coli as described previously [15]. We first generated a transfer vector in which 49 bp of the Bm61 locus region were replaced with a Cm resistance cassette for antibiotic selection in E. coli. A 500 bp 5’-flanking region of Bm61 was PCR amplified from the BmNPV bacmid using the primers 61-US-F and 61-US-R (Supplementary Table S1). The PCR product was digested with EcoRI and BamHI, and then ligated into vector pUC18-Cm to generate the recombinant plasmid pUC18-US-Cm. With primers 61-DS-F and 61-DS-R (Supplementary Table S1), a 497 bp 3’-flanking region of Bm61 was similarly amplified from the BmNPV bacmid. The PCR product was digested with PstI and HindIII, and cloned into plasmid pUC61-US-Cm that was digested with PstI and HindIII to generate a final Bm61 knockout transfer vector named pUC61-US-Cm-DS. This transfer vector was digested with EcoRI and BamHI, and the resulting linear 2017 bp fragment containing the Cm gene cassette and Bm61 flanking regions was gel purified and resuspended in distilled water to a final concentration of 200 ng/μl. The Bm61 knockout bacmid was named vBm61-kd.

A further knockout bacmid (vBm61-ko) constructs was prepared by transposition with the pFB1-gfp-polh transfer vector according to the methods described previously [16] to introduce the gfp reporter gene under control of the BmNPV i.e.-1 promoter. The *polyhedrin* gene (polh) coding sequence plus polyadenylation signal was amplified from BmNPV DNA by PCR using the primer pair PH–F and PH–R (Supplementary Table S1); the *polyhedrin* promoter derived from pFastBac1. To construct a repair bacmid (vBm61-re), an 828 bp fragment containing the Bm61 gene with its native promoter and polyadenylation signal was PCR amplified using primers Repair-F and Repair-R (Supplementary Table S1). The repair fragments were cloned into the pFB1-gfp-polh plasmid to give pFB1-61-gfp-polh and used to transpose parental knockout bacmids. The control virus (vBm-wt) was constructed by transposing the bacmid with the pFB1-gfp-polh plasmid.

PCR analysis was used to confirm the absence of the Bm61 gene in the BmNPV bacmid and its replacement by the Cm gene. Primers FA-U and FA-D (Supplementary Table S1), which are just outside the flanking sequence for recombination, were used to further confirm the deletion region. Primer pairs FA-U/FA-D, FA-U/CmD and CmU/FA-D (Supplementary Table S1) were used to detect the correct insertion of the Cm gene cassette.

### 2.3. Analysis of virus growth curve

1 × 10⁶ BmN cells were transfected with 2.0 μg of vBm-wt, vBm61-ko, and vBm61-re bacmid DNA, respectively. Virus supernatant was collected at 12, 24, 48, 72 and 96 hpt (hours post-transfection). The titers of BV were determined by tissue culture infective dose (TCID₅₀) end-point dilution assay on BmN cells [17].

### 2.4. DNA replication and BV titration analysis by real-time quantitative PCR (qPCR)

The qPCR assay was performed as described previously [18]. To prepare total DNA for analysis, 1 × 10⁶ BmN cells were transfected with vBm-wt, vBm61-ko or vBm61-re, and at 12, 24, 48, 72 and 96 hpt, virus DNA was extracted as previously described [19]. Prior to PCR, 5 μl of total DNA from each time point was digested with 2U of *DpnI* restriction enzyme (Fermentas) overnight in 20 μl total reaction volume. The primers P–F and P–R (Supplementary Table S1) were used to amplify a 101 bp region within the gfpI gene of BmNPV. A standard curve was created with a twofold sample of purified vBm-wt DNA templates. Five dilutions (each 1:10) were prepared to cover the workable concentrations of the DNA templates.

The use of qPCR to titrate baculovirus stocks has been previously described [20,21]. Primers IE-1-F and IE-1-R (Supplementary Table S1) were designed to amplify a 315 bp genomic fragment of IE-1. BmN cells (2.0 × 10⁶/35-mm-diameter six-well plate) were transfected with 2 μg of each bacmid (vBm61-ko and vBm61-re). At 24, 48 and 72 hpt, to prepare DNA for analysis supernatant containing BV was harvested and cell debris was removed by centrifugation (8000 × g for 5 min). An aliquot of each of these supernatants (100 μl) was processed using the AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit. The purified DNA was eluted in 30 μl of elution buffer, and 8 μl of sample was used for qPCR analysis.

### 2.5. Localization of Bm61 in BmN cells

The polyhedrin promoter was removed from pFastBac1 to create pFB1-ph . The Bm61 gene without the stop codon (TA) was amplified from BmNPV bacmid with the PCR primers LO-F and LO-R (Supplementary Table S1). The PCR product was inserted into pFB1-ph to generate pFB1-Bm61. The gfp gene was cloned into pFB1-Bm61 to generate pFB1-Bm61-gfp and used to transpose BmNPV bacmids. Thus, Bm61 is expressed with a gfp tag under the control of the Bm61 native promoter in the resulting bacmid, which is referred to as vBmBm61-gfp. The control bacmid vBm61gfp, in which only gfp is expressed under the control of the Bm61 native promoter, was generated in a procedure similar to that for vBm61-gfp.

### 2.6. Transmission electron microscopy (TEM)

BmN cells (2 × 10⁶) were transfected with 1.0 μg of vBm61-ko and vBm61-re bacmid, respectively. At 36 and 96 hpt, the supernatant was removed and the cells were washed once with PBS (pH 7.2) and then fixed in 2.5% glutaraldehyde in PBS for 15 min. Cells were then dislodged with a rubber policeman, transferred into Eppendorf tubes, and pelleted at 2000 × g for 5 min. Cells were fixed, dehydrated, embedded, sectioned, and stained as described previously [22].

### 3. Results

#### 3.1. Construction of knockout, wt and repair bacmids containing polyhedrin and gfp

To determine whether the Bm61 gene is essential for viral replication, deletion and repair constructs were prepared. The vBm61-ko bacmid constructs were selected by their resistance to chloramphenicol, which indicated that site specific deletion of the target gene had occurred. vBm61-ko, containing polyhedrin and gfp genes, was constructed by transposition of *polyhedrin* and *gfp* genes into the *polyhedrin* locus of vBm61-ko, using the Bac-to-Bac system (Supplementary Fig. S1A). The repair bacmid, vBm61-re, was generated by inserting Bm61 under the control of its own promoter as well as *polyhedrin* and *gfp* into the *polyhedrin* locus by transposition. *polyhedrin* and *gfp* were also inserted into the *polyhedrin* locus of the BmNPV bacmid to generate a wt bacmid named vBm-wt, which was used as a positive control (Supplementary Fig. S1A). The structure of all the deletion and repaired constructs is shown in Supplementary...
Fig. S1A. Constructs were confirmed by PCR (Supplementary Fig. S1B). Positions of primer pairs are shown in Supplementary Fig. S1C.

3.2. Analysis of deletion, wt and repair bacmid replication in transfected BmN cells

At 96 hpt, GFP fluorescence was observed in almost all vBm-wt and vBm61-re transfected cells (Fig. 1A), indicating that vBm-wt or vBm61-re generated infectious budded virions from the initial transfection. In contrast, vBm61-ko transfected cells showed few fluorescent cells (Fig. 1A), suggesting that there was no spread of the virus beyond the cells transfected with the Bm61 deletion bacmid DNA. In order to verify the lack of secondary infection after transfection with the Bm61 deletion bacmid, supernatant was collected from the all initial transfected cultures at 96 hpt, and used to infect new cultures. At 72 h post infection (hpi), fluorescence was observed in almost all cells in secondary cultures derived from the vBm-wt or vBm61-re-transfection, but not those from Bm61 knockout infected cells (Fig. 1A). These results showed that the deletion of Bm61 leads to a defect in BV production or infection. Light microscopy analysis showed that, at 96 hpt, no occlusion bodies were found in the vBm61-ko transfected cells (Fig. 1B). In contrast, at 96 hpt in vBm-wt and vBm61-re-transfected cells, almost every cell contained occlusion bodies indicating production of BV and spread of the infection (Fig. 1B).

We further determined the effect of deleting Bm61 on replication kinetics of virus. BmN cells were transfected with bacmid DNAs, and at selected time points thereafter the BV titers were determined by a TCID_{50} end-point dilution assay. BmN cells transfected with vBm-wt or vBm61-re revealed a steady increase in virus production (Fig. 1C). In contrast, no virus was detectable at any time point up to 96 hpt for vBm61-ko transfected cells (Fig. 1C).

3.3. Bm61 deletion does not affect viral DNA replication but blocks BV production

To detect whether the deletion of Bm61 affects the viral DNA replication, a qPCR analysis was performed to compare the initiation and viral DNA replication level among vBm-wt, vBm61-re, or vBm61-ko bacmid transfected cells. For all three constructs, replicated DNA was detectible at similar levels at 24 hpt (Fig. 2A). The DNA levels generated by vBm61-re and vBm-wt-transfected cells showed a similarly steady increase from 24 to 96 hpt (Fig. 2A). However, for vBm61-ko transfected cells there was no evident increase from 24 to 96 hpt (Fig. 2A). Based on the equal numbers of cells collected at designated time points, these data suggested that the increase of DNA levels generated by vBm61-re or vBm-wt was due to the secondary infection, whereas the replication of vBm61-ko DNA was restricted primarily to the initially transfected cells. Therefore, qPCR analysis suggests that Bm61 is not required for the onset of viral DNA replication in BmN cells.

The TCID_{50} end-point dilution assays determine the production of infectious BV, but cannot assay if any non-infectious BVs are produced. To further analyze this possibility, the BV titers of our virus constructs were also determined by qPCR analysis, which detects viral genomes regardless of infectivity. Due to bacmid transfection there is detectable background of viral genomes present at the 24 and 72 hpt time points analyzed. As expected for vBm61-re, a strong increase in BV DNA was detected at 72 hpt. In contrast, for vBm61-ko-transfected cells no increase in BV DNA was detected above background at 72 hpt (Fig. 2B). The qPCR assay was therefore unable to detect any BV production by the Bm61 knockout, in agreement with the TCID_{50} results.

3.4. Cellular localization of BM61 in BmN cells

To assess the subcellular localization of Bm61, two recombinant baculoviruses, vBmBM61-GFP and vBmBM61p-GFP were constructed. GFP was fused to the C terminus of Bm61 and expressed under the control of the Bm61 native promoter in vBmBM61-GFP. As a control, GFP alone was expressed under the control of the Bm61 native promoter in vBmBM61p-GFP (Fig. 3A). BmN cells infected with vBmBM61p-GFP and vBmBM61-GFP at an MOI of 10 were examined for GFP specific fluorescence by laser confocal microscopy (Fig. 3B). The localization of BM61-GFP from vBmBM61-GFP infected cells was observed. Fluorescence accumulated at the nuclear membrane and ring zone of infected cells until 48 hpi. However, in the negative controls, GFP showed homogeneous fluorescence in the cytoplasm and nucleus when infected with vBmBM61p-GFP (Fig. 3B). These results suggest that BM61 localizes in the nuclear membrane and membrane ring zone of infected cells.

3.5. Electron microscopic analysis of Bm61 deletion and repair bacmid transfected cells

TCID_{50} results indicated that Bm61 is essential for BV production. The last essential steps in the BmNPV BV life cycle are the

Fig. 1. Analysis of viral replication in BmN cells. (A) The upper row of panels shows GFP fluorescence of BmN cells transfected with vBm61-ko, vBm-wt and vBm61-re, at 96 hpt. Supernatants from such cultures were used for secondary passage in BmN cells, which were analyzed at 72 hpi (B) The presence of occlusion bodies was assessed at 96 hpt. (C) Virus growth curves generated from transfection of BmN cells. BmN cells were transfected with 2 μg of bacmid DNA of each type. Cells culture supernatants were harvested at the selected time points and the production of infectious virus assessed by TCID_{50} assay.
assembly of nucleocapsids and egress from the nucleus. Electron microscopic analysis was used to further analyze whether the deletion of Bm61 interferes with the assembly and egress of nucleocapsids. BmN cells were transfected with either vBm61-k0 or vBm-re DNA, and examined by TEM at 36 hpt. Nuclei of cells transfected with either vBm61-k0 or vBm-re DNA exhibited the typical baculovirus infection symptoms such as an extensive number of nucleocapsids, enlargement of the nucleus, and reorganized, electron dense virogenic stroma (Fig. 4A,B). No obvious difference between the nucleocapsids of vBm61-k0 and vBm-re viruses was observed, indicating that Bm61 is not required for the assembly of nucleocapsids. In most cells transfected with the vBm61-k0 virus, nucleocapsids were found in the nucleus, but nucleocapsids were never found (0 of 63 cells) to be closely associated with or passing through the nuclear membrane and into the cytoplasm, or budding at the cytoplasmic membrane (Fig. 4D). In contrast, cells transfected with vBm-re virus showed many nucleocapsids penetrating the nuclear membrane, residing in the cytoplasm, and budding (Fig. 4C). These observations indicated that Bm61 is not required for the assembly of nucleocapsids, but rather is required for the egress of nucleocapsids from the nucleus. At 72 hpt, no occlusion bodies were found in the vBm61-k0 transfected cells (Fig. 4F), but existed in vBm-wt and vBm61-re transfected cells (Fig. 4E). These observations further support the idea that Bm61 is required for the egress of nucleocapsids from the nucleus.

4. Discussion

Bm61 is a highly conserved gene in lepidopteran NPVs, and sequence-based queries performed with the InterProScan program show that Bm61 homologues constitute a protein family (DUF1160) of unknown function. This suggests that BM61 and its homologues might play an important role in baculoviridae infection cycles. In this report, BV production was shown to be completely blocked by deletion of Bm61 from BmNPV bacmid. Electron microscopic analysis was employed to investigate the role of Bm61. Cells transfected with Bm61 knockout bacmid exhibited normal assembly of nucleocapsids, but the nucleocapsids were not able to egress from the nucleus and migrate through the cytoplasm, leading to loss of BV production. Rescue of the repair virus phenotype by reinsertion of Bm61 into the polyhedrin locus of the deletion bacmid confirmed that the observed phenotype was directly due to the deletion of Bm61 from BmNPV bacmid DNA. BV production is a complex procedure that involves many viral and cellular proteins. Thus far, how BV are produced is not clear. In previous studies, several viral genes have been shown to be involved in BV production. For example the P78/83 [23], 38 k [15,24], VLF-1 [25] and VP1054 [26] proteins were reported previously as nucleocapsid proteins, and these proteins are involved in the nucleocapsid packaging; ac66 and EXON0 are not essential for the efficient transport of nucleocapsids from the nucleus to the...
cytoplasm [27,28]; P48(ac103) is required for nucleocapsid envelopment in BV production [29]. Thus far no mechanism by which nucleocapsids enter the cytoplasm from the nucleus has been proposed. It has been shown that nucleocapsids may exit the nucleus through nuclear pores, the nuclear membrane, or via migration into endoplasmic reticulum [30–32]. Exiting through the nuclear membrane is the most common method of egress observed in electron microscopic studies, by which nucleocapsids acquire a double membrane vesicle derived from the nuclear membrane [32].

Transfection-infection tests showed that production of BV is blocked by deletion of Bm61, although DNA replication is initiated. These results therefore suggest that Bm61 executes its function after DNA replication, and at or prior to the BV budding process. GFP-tagged BM61 was located in intranuclear ring zone and nuclear membrane, suggesting that Bm61 might facilitate the transport of nucleocapsids to the cytoplasm. A BV-associated protein, orf68 of BmNPV, was also found to be localized to the nuclear membrane [33]. Similar protein localization was also found for some nucleocapsid proteins, such as BV/ODV-C42 and EXON0 [34,35]. However, EXON0 knockout did not affect nucleocapsid morphogenesis. The previous studies showed that nucleocapsid morphogenesis and polyhedron development occur in the intranuclear ring zone

Fig. 4. Electron microscopic analysis of BmN cells transfected with either Bm-wt (A, C, E) or Bm61-ko (B, D, F) at 36 hpt (A, C, B, D) and 72 hpt (E, F). The nucleocapsids (NC), nuclear membrane (NM), ring zone (RZ), cytoplasm (Cyt), polyhedron (P) and virogenic stroma (VS) regions are indicated. Panels C and D are of higher-magnification images of the boxed regions in panels A and B, respectively. Nucleocapsids in the cytoplasm or budding are indicated with short arrows. Longer black arrows indicate nucleocapsids in the virogenic stroma and ring zone. White arrows indicate ODV in the ring zone. Scale bars. 2 µm in A–B, 0.5 µm in C, 1 µm in F, 2 µm in E–F.
Our data indicate that Bm61 is required for egress and transport of nucleocapsids across the nuclear membrane.

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Appendix A. Supplementary data


References