Analysis of BmNPV *orf101* disruption: *orf101* is essential for mediating budded virus production

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Received: 10 May 2014 / Accepted: 9 July 2014 / Published online: 10 October 2014
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Abstract In our previous study, *Orf101* (*Bm101*) of *Bombyx mori* nucleopolyhedrovirus (BmNPV) was identified as a component of the budded virions important for viral late gene expression. In this study we demonstrate that *Bm101* is actually a previously unrecognized core gene and that it is essential for mediating budded virus production. To determine the role of *Bm101* in the baculovirus life cycle, a *Bm101* knockout bacmid containing the BmNPV genome was generated through homologous recombination in *Escherichia coli*. Furthermore, a *Bm101* repair bacmid was constructed by transposing the *Bm101* open reading frame with its native promoter region into the polyhedrin locus of the *Bm101* knockout bacmid. Bacmid DNA transfection assay revealed that the *Bm101* knockout bacmid was unable to produce the infectious budded virus, while the *Bm101* repair bacmid rescued this defect, allowing budded-virus titers to reach wild-type levels. Real time PCR analysis indicated that the viral DNA genome in the absence of *Bm101* was unaffected in the first 24 h p.t. Thus, studies of a *Bm101*-null BACmid indicate that *Bm101* is required for viral DNA replication during the infection cycle.

Keywords *Bombyx mori* nucleopolyhedrovirus · *Orf101* · Knockout

Introduction

Baculoviruses are a very diverse group of viruses with double-stranded, circular, supercoiled genomes, with sizes varying from about 80 to over 180 kb and that encode between 90 and 180 genes. In the most well characterized baculoviruses, the virions are present as two types, occluded virions (ODV) and budded virions (BV; Herniou et al. 2003). BV and ODV share dozens of the same proteins, in which some proteins are essential for nucleocapsid formation including P6.9 (Wang et al. 2010), VP39 (Thiem and Miller 1989) and 38K (Wu et al. 2006). Although the nucleocapsids are similar in the two, the envelopes differ in composition, reflecting their different functions during infection. The virions have distinct tissue tropism: ODVs infect midgut epithelial cells up to 10,000-fold more efficiently than do BVs, whereas BVs infect cultured cells 1,000-fold more efficiently than do ODVs. Thus, these two virus forms play significantly different roles in the virus life cycle (Granados and Lawler 1981; Keddie et al. 1989). In our previous study, it was shown that *Bombyx mori* nucleopolyhedrovirus (BmNPV) *orf101* (*Bm101*) is a functional ORF of BmNPV and encodes a protein expressed in the late stage of the infection cycle that is associated with the BV envelope (Chen et al. 2014).

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Various nucleopolyhedrovirus genes have been shown to affect the production of BV and ODV. Core gene GP64 is a structural component of BV and is required for the efficient virion budding from the plasma membrane and is essential for BV attachment and membrane fusion (Blissard and Wenz 1992; Hefferon et al. 1999). Gp41 (ac80) is also a core gene that expresses an O-glycosylated protein which affects both BV and ODV but is only a structural component of ODV. Me53, associated with both BV and ODV, is essential for DNA replication and important for efficient BV production (de Jong et al. 2009). The baculovirus core gene 38K (ac98) plays a role in nucleocapsid assembly, affecting both BV and ODV production, but it has not been shown to be a structural component of nucleocapsids (Wu et al. 2006). In this report we provide additional information on the role Bm101 plays in baculovirus biology. To accomplish this we generated *Bm101* knockout bacmid as well as repair bacmid constructs by homologous recombination in *Escherichia coli*. These constructs were then characterized in a tissue culture system to determine the role of Bm101 in virus infectivity and DNA replication.

Many BV envelope proteins have been identified, such as GP64, F-like protein, V-ubiquitin, GP37, ODV-E25, ODV-E18 and BV/ODV-E26, and the functions of several of them have been investigated by deletion of the gene (Monsma et al. 1996; Wang et al. 2008; McCarthy and Theilmann 2008). Bm101, which has been identified as a structural protein of BV envelope, is poorly understood because no gene deletion mutants have been generated (Chen et al. 2014). In this report, we generated a BmNPV bacmid in which *orf101* was disrupted by homologous recombination and investigated the role of Bm101 in BmNPV infection of BmN cells using the *Bm101* null virus. The infectivity of the *Bm101*-deletion virus was significantly reduced compared to that of the wild-type (wt) virus or the repaired virus.

Materials and methods

Cells and virus

The BmN cell line was maintained in TC-100 (Applchem, Darmstadt, Germany) with 10 % fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 29 °C. The *E. coli* strains BW25113 (pKD46; encoding genes for the λ Red system), BW25141 [pKD3; encoding a

chloramphenicol resistance gene (*Cm*)], and the cell line DH10B were kept in our lab. The *E. coli* strain DH10B was used to isolate the bacmid containing the BmNPV genome and the helper plasmid pMON7124 encoding a transposase (Luckow et al. 1993). In all experiments, the virus inoculum was allowed to adsorb for 1 h upon infection or 5 h upon transfection at 29 °C and then was replaced with fresh medium. Time zero was defined as the time when the virus was replaced with fresh medium.

Construction of the *Bm101* knockout BmNPV bacmid

To knockout the *orf101* from BmNPV bacmid, we generated a transfer vector in which *Bm101* was replaced by *Cm* for antibiotic selection in *E. coli*. The construction strategy of *Bm101*-null bacmid was constructed in a manner similar to protocols described previously (Lung et al. 2003). First, a 1,031 bp *Bam*HI-*Pst*I fragment containing *Cm* was amplified from plasmid pKD3 with primers CmF (AGGATCCTGTGTAGGCTGGAGCTGC) and CmR (ACTGCAGTCCATATGAATATCCTCC). The PCR product was digested with *Bam*HI and *Pst*I and ligated into vector pUC18 to generate the recombinant plasmid pUC18-*Cm*. Second, an 806 bp fragment homologous to the upstream sequence of *Bm101* (BmNPV nucleotides 94879–95667) was amplified from the BmNPV genome with primers ORF101UF (AGAATTCGTGCTGAATTAA TTT) and ORF101UR (CGGATCCTTAGTCGGTCGATAA), digested with *Eco*RI and *Bam*HI, and then ligated with pUC18-*Cm* which was digested with *Eco*RI and *Bam*HI to generate pUC18-*Cm*U. Third, with primers ORF101DF (GCTGCAGAATTTACAAAGTATT) and ORF101DR (CAAGCTTAAGTTGACACCTGCC), a 572 bp *Pst*I-*Hind*III fragment homologous to the downstream sequence of *Bm101* (BmNPV nucleotides 96259–96846) was also amplified. The PCR product was digested with *Pst*I and *Hind*III and cloned into plasmid pUC18-*Cm*U, which was digested with *Pst*I and *Hind*III to generate a final *Bm101* knockout transfer vector named pUC18-*Cm*UD. The plasmid was verified by PCR, restriction analysis and DNA sequencing. This transfer vector was digested with *Eco*RI and *Hind*III, and the resulting linear 2.4 kb fragment containing the *Cm* gene cassette and *Bm101* flanking region was gel purified and suspended in water.

DH10B cells containing the BmNPV and pKD46 were made electrocompetent using a method

described previously (Pijlman et al. 2002). The DNA fragment was electroporated into the the λ Red system-induced cells. Recombinant bacmid was obtained and named vBm^{101-KO}.

PCR confirmation of recombinant bacmids

Purified bacmids were screened to confirm the replacement of the *Bm101* with the *Cm* cassette by PCR with different primer sets. Primers ORF101F (ATGGGTT TGTTCACG) and ORF101R (TTACTTTTATTTTT) were used to confirm the expected deleted region and the insertion of *Cm* at the *Bm101* locus. Primers ORF101UF and ORF101DR, which bind to the sequences outside the region of recombination, were used to confirm the deletion region. Primers CatF (TCACCGTCTTTCATTGCCAT) and CatR (TTGTT ACACCGTTTTCCATGAGC) were specific for the *Cm* gene. The primer combinations ORF101UF + CatR and ORF101DR + CatF generated PCR products from the *Bm101* knockout bacmid, but were unable to amplify PCR products from the wt bacmid.

Construction of knockout, repair, and wt BmNPV bacmids containing *polyhedrin* and *egfp*

To determine if *Bm101* deletion has any effect on occlusion morphogenesis and to facilitate examination of virus infection, *polyhedrin* and an enhanced green fluorescence protein gene (*egfp*) were inserted into the *polyhedrin* locus by site specific transposition. *Polyhedrin* and *egfp* were inserted into the pFastBacHtb plasmid under the control of the *polyhedrin* promoter and BmNPV *ie1* promoter, respectively, to generate the donor plasmid pFB-PH-EGFP according to the method described by Dai et al. (2004). To construct repair bacmid, gene fragments were PCR amplified from bacmid DNA using 5' primers 101rescuedF (GCTCTA GACATTTTCGGGGGTGTAC) that annealed ~500 bp upstream of the ATG to include the native promoter region along with 3' primers 101rescuedR (GCGTCGACTTACTTTTTATTTTTATG) that contained the poly (A) tail. The PCR product was inserted into pFB-PH-GFP, which was digested with *XbaI* and *SalI*, and named pFB-101-PH-EGFP. pFB-PH-EGFP and pFB-101-PH-EGFP were transformed to electrocompetent DH10B cells containing helper plasmid and vBm^{101-KO}, to generate *orf101*-null bacmid vBm^{101-KO-PH-EGFP}, and *orf101* repair bacmid vBm^{101-REP-PH-EGFP},

respectively. Electrocompetent DH10B cells containing helper and the bacmid were transformed with pFB-PH-EGFP to give a wt control virus named vBm^{PH-EGFP}.

Virus growth curve

BmN cells were transfected in triplicate with the appropriate bacmid DNA (vBm^{101-KO-PH-EGFP}, vBm^{101-REP-PH-EGFP}, vBm^{PH-EGFP}) using Cellfectin liposome reagent (Invitrogen Life Technologies) or infected in triplicate with the supernatant of vBm^{101-REP-PH-EGFP} or vBm^{PH-EGFP} at multiplicity of infection (MOI) of five. Virus supernatants were harvested at various times post-transfection (p.t.) or post-infection (p.i.). BV production was determined by a 50 % tissue culture infective dose (TCID₅₀) endpoint dilution assay on Sf9 cells (O'Reilly et al. 1992).

DNA replication analysis by real-time PCR

To assess viral DNA replication, a quantitative real-time PCR assay was performed. Two primers ORF104-99226 (TAATCTGTCTCGGAGCAGCAAA) and ORF104-99475 (ACAATGTCTGGCAGCGTC TAT) were designed on the basis of the sequence of the BmNPV *orf104*, corresponding to BmNPV genomic coordinates nt 99226–99205 and nt 99475–99496, respectively. BmN cells were transfected at the indicated time point and the cells were harvested in 0.5 ml PBS, lysed in 200 μ l cell lysis buffer, and incubated overnight at 60 °C. DNA was extracted by phenol/chloroform extraction, precipitated using ethanol, and suspended in 50 μ l of water. Quantitative PCR was performed with 2 μ l of the DpnI-digested DNA added to SYBR® Premix Ex Taq™ (perfect real time) kit (Takara, Dalian, China) according to the manufacturer's instructions. The thermal profile used was as follows: 1 cycle of 95 °C for 15 min; 40 cycles of 95 °C for 30 s, 56 °C for 24 s, 72 °C for 30 s; 1 cycle of 95 °C for 1 min; 41 cycles of 55 °C for 30 s.

Results

Construction of *Bm101*-knockout and rescued BmNPV bacmids

A *Bm101* gene knockout was constructed by deleting the central portion of *Bm101* and retaining 225 nt from

Fig. 1 Construction of recombinant bacmids. **a** Strategy for construction of *Bm101* deletion. *Bm101* fragment (414 nt) was deleted and replaced with the chloramphenicol resistance gene (*Cm*) between nt 95845 and nt 96259 of the BmNPV genome. **b** PCR confirmation of *Bm101* deletion. The table below the agarose gel shows the templates, primers, and expected product sizes. *NA* no amplified product expected. **c** Schematic diagram

of vBm^{PH-EGFP} showing the polyhedrin (*polh*) and enhanced green fluorescent protein (*egfp*) genes inserted into the polyhedrin locus by Tn7-mediated transposition. **d** Schematic diagram of vBm^{101-KO-PH-EGFP} and vBm^{101-REP-PH-EGFP}, showing *polh* and *egfp* inserted into the polyhedrin locus after Tn7-mediated transposition

the 5' end and 93 nt from the 3' end of the *Bm101* coding region to ensure the expression of the neighbouring genes *Bm100* and *Bm102* (Fig. 1a). The remaining 414-nt coding sequence (nt 95845–96259 of BmNPV) was replaced with the *Cm* cassette (Fig. 1a).

The deletion of *Bm101* from the *Bm101* locus of BmBac and the insertion of the *Cm* cassette were confirmed by PCR (Fig. 1b). Primers ORF101F and ORF101R were used to confirm the deletion of 414 bp within the *Bm101* coding region and its replacement with the 1,017 bp *Cm* cassette. Primers pairs ORF101UF/CatR and CatF/ORF101DR were used to confirm the recombination junctions upstream and downstream of *Bm101*, respectively. The sizes of the PCR-amplified products obtained were as predicted following successful recombination (Fig. 1b).

To investigate the function of *Bm101* during the viral infection cycle, we generated a *Bm101* gene knockout BmNPV mutant bacmid (vBm^{101-KO-PH-EGFP}) by using an efficient RecA-mediated homology recombination

technology. And to rescue the defect of function resulting from the *Bm101* gene knockout, we also generated a repaired bacmid (vBm^{101-REP-PH-EGFP}) from the mutant by reinsertion of the *Bm101* gene into the mutant with ET-recombination technique at a different locus. To facilitate the observation of virus transmission among the cell cultures, *egfp* and *polyhedrin* genes were introduced into the bacmid as two markers, so that Sf9 cells transfected with vBm^{101-KO-PH-EGFP}, vBm^{101-REP-PH-EGFP}, and vBm^{PH-EGFP}, respectively, were able to be examined for viral propagation with ease by the phenotype of EGFP expression and occlusion body formation. The structure of the *Bm101* construct is shown in Fig. 1a and confirmed by PCR (Fig. 1b). Primers CatF and ORF101DR produced no PCR product in vBm^{PH-EGFP}, produced a single 1,104 bp fragment in vBm^{101-KO-PH-EGFP} and vBm^{101-REP-PH-EGFP} (Fig. 1b). Furthermore, primers ORF101UF and CatR produced no PCR product in vBm^{PH-EGFP}, but a 1263 fragment in

Fig. 2 Analysis of viral replication by the *Bm101* knockout virus in BmN cells. **a** Fluorescence microscopy of *vBm*^{101KO-PH-EGFP}, *vBm*^{101-REP-PH-EGFP}, and *vBm*^{PH-EGFP}-transfected BmN cells at 24, 48 and 72 h p.t. **b** Light microscopy of *vBm*^{101KO-PH-EGFP}, *vBm*^{101-REP-PH-EGFP} and *vBm*^{PH-EGFP}-transfected BmN cells at 96 h p.t. **c** Virus growth curves generated from either transfected cells or infected cells. For the transfection growth curves, BmN cells were transfected with each bacmid DNA, and then the supernatants were harvested at the indicated time points posttransfection and the titers were determined by TCID₅₀ assay. For the infection growth curves, cells were infected at an MOI of five for each virus, and cell culture supernatants were harvested at the selected time points and assayed for the production of infectious virus by TCID₅₀ assay. Each time point was determined from the average of three independent transfections or infections and *error bars* represent the SE

Bm101-null bacmid (Fig. 1b). Tn7-mediated transposition was also confirmed by PCR with M13 primers (Fig. 1b). The results indicated that *egfp* and *Bm101* under the control of their own promoter had been transposed correctly into the polyhedrin locus.

To facilitate an examination of the effects of the deletion of *Bm101* on virus infection, a *Bm101*-knockout mutant, $vBm^{101-KO-PH-EGFP}$, containing the polyhedrin gene under polyhedrin promoter control and *egfp* under *i.e.-I* promoter control, was constructed by the transposition of the *polyhedrin* and *egfp* genes into the polyhedrin locus of vBm^{101-KO} (Fig. 1d). The repair bacmid was constructed to rescue and confirm the phenotype resulting from the deletion of *Bm101*, $vBm^{101-REP-PH-EGFP}$, carrying the *Bm101*, *polyhedrin*, and *egfp* genes (Fig. 1d). $vBm^{PH-EGFP}$, BmBac carrying the *polyhedrin* and *egfp* genes, was used as a positive control (Fig. 1c).

Analysis of virus propagation in BmN cells by the *Bm101* knockout bacmid

To determine whether the *Bm101* is essential for viral replication, BmN cells were transfected with recombinant bacmid $vBm^{101-KO-PH-EGFP}$, $vBm^{101-REP-PH-EGFP}$ or $vBm^{PH-EGFP}$, respectively. Infected cells were monitored for GFP expression by fluorescence microscopy (Fig. 2a). No differences were observed among these three viruses at 24 h p.t. and the comparable transfection efficiencies were approximately 10 % (Fig. 2a). Widespread fluorescence was observed in $vBm^{101-REP-PH-EGFP}$ - and $vBm^{PH-EGFP}$ -transfected cells by 48 h p.t. (Fig. 2a), indicating that the wt and repair bacmids were able to produce infectious BV to initiate secondary infection. In contrast, there was almost no increase in the number of fluorescent cells in $vBm^{101-KO-PH-EGFP}$ -transfected cells (Fig. 2a), suggesting that the *Bm101* knockout bacmid may be unable to produce infectious BVs to initiate secondary infection.

Microscopic analysis showed that occlusion bodies were observed in $vBm^{101-KO-PH-EGFP}$ -transfected cells as well as in $vBm^{101-REP-PH-EGFP}$ - and $vBm^{PH-EGFP}$ -transfected cells. At 48 h p.t., there were no differences among these three bacmids in the number of cells containing occlusion bodies (data not shown). However, by 96 h p.t., significant differences were observed between $vBm^{101-KO-PH-EGFP}$ and $vBm^{101-REP-PH-EGFP}$ or $vBm^{PH-EGFP}$. Most of the $vBm^{101-REP-PH-EGFP}$ - and

$vBm^{PH-EGFP}$ -transfected cells contained occlusion bodies but the number of the $vBm^{101-KO-PH-EGFP}$ -transfected cells containing occlusion bodies was small (Fig. 2b). The polyhedra are observed only in the GFP-positive cells transfected with $vBm^{101-KO-PH-EGFP}$, suggesting that infection is really limited to the cells initially transfected. In a minority of GFP-positive cells, polyhedra did not appear even at 96 h p.t., indicating that the infection was unable to progress to the very late phase. Nevertheless, the bacmid transfection experiments showed that deletion of $vBm^{101-KO-PH-EGFP}$ leads to a defect in the production of infectious BVs and occlusion body morphogenesis.

To further determine the effect of *Bm101* deletion on virus replication, a virus growth curve analysis was performed (Fig. 2c). Two separate growth curve experiments are shown in Fig. 2c. In the first experiment, BmN cells were transfected with $vBm^{101-KO-PH-EGFP}$, $vBm^{101-REP-PH-EGFP}$ and $vBm^{PH-EGFP}$. At time points selected, the BV titers were determined by a TCID₅₀ end-point dilution assay. There was no virus titer detected in the supernatant from $vBm^{101-KO-PH-EGFP}$ -transfected cells at any time point up to 120 h p.t. (Fig. 2c). In contrast, the supernatant from BmN cells transfected with $vBm^{101-REP-PH-EGFP}$ or $vBm^{PH-EGFP}$ revealed a steady increase in virus production (Fig. 2c). To further determine whether the *Bm101* repair bacmid was able to rescue the defect in production of progeny virus, a second growth curve was performed with the supernatants prepared from cells infected with BVs obtained from $vBm^{101-REP-PH-EGFP}$ - and $vBm^{PH-EGFP}$ -infected cells at a MOI of five. The *Bm101* repair virus showed similar growth kinetics in titer to the wt virus (Fig. 2c). Thus, these data showed that the defect in virus production in *Bm101*-null virus was due solely to the deletion of *Bm101*, as the defect can be rescued by insertion of the *Bm101* gene into the polyhedrin locus of the *Bm101*-null bacmid. They also confirmed that *Bm101* is essential for virus replication.

Western blot analysis of $vBm^{101-KO-PH-EGFP}$ -transfected cells

Microscopic analysis and viral growth curves showed that no infectious BVs were produced in cells transfected with $vBm^{101-KO-PH-EGFP}$. There may be two possibilities: (i) no BVs budded from the infected cells; (ii) normal levels of BVs were produced but they were non-infectious. To address this issue, BVs were

Fig. 3 Western blot analysis of purified BV particles. Viral particles were purified from supernatants of BmN cells that had been transfected with vBm^{101-KO-PH-EGFP}, vBm^{101-REP-PH-EGFP}, vBm^{PH-EGFP}

or vBm^{PH-EGFP}. Samples were analyzed with two monoclonal antibodies specific for nucleocapsid protein VP39 and BV-specific protein GP64

purified from bacmid transfected cell supernatants and analyzed by Western blot to compare the levels of nucleocapsid protein VP39 and envelope protein GP64. Similar levels of GP64 and VP39 were detected from vBm^{101-REP-PH-EGFP}- and vBm^{PH-EGFP}-transfected cells. Analysis of vBm^{101-KO-PH-EGFP}-transfected cell supernatant failed to detect any envelope protein GP64 even with a longer exposure time (Fig. 3). The VP39 results were very similar, except that a very faint signal was detected in vBm^{101-KO-PH-EGFP}, but in comparison to vBm^{101-REP-PH-EGFP} or vBm^{PH-EGFP} the levels were drastically reduced (Fig. 3). Taken together, these results show that BV particles are at extremely low levels in vBm^{101-KO-PH-EGFP}-transfected cell culture, indicating that deletion of *Bm101* leads to a defect in BV production.

Bm101 deletion does not affect onset of viral DNA replication but blocks BV production

To detect whether the deletion of *Bm101* affects the viral DNA replication, a qPCR analysis was performed to compare the initiation and viral DNA replication level among vBm^{101-KO-PH-EGFP}, vBm^{101-REP-PH-EGFP}, or vBm^{PH-EGFP} bacmid transfected cells. For all three constructs, replicated DNA was detectable at similar levels from 12 to 24 h p.t. (Fig. 4a). The DNA levels generated by vBm^{101-REP-PH-EGFP}- and vBm^{PH-EGFP} transfected cells showed a similarly steady increase from 24 to 96 h p.t. (Fig. 4a). However, for vBm^{101-KO-PH-EGFP}-transfected cells there was no evident increase from 24 to 96 h p.t. (Fig. 4a). Based on the equal numbers of cells collected at designated time points, these data suggested that the increase of DNA levels generated by vBm^{101-REP-PH-EGFP} or vBm^{PH-EGFP}-transfected cells

was due to the secondary infection, whereas the replication of vBm^{101-KO-PH-EGFP} DNA was restricted primarily to the initially transfected cells. Therefore, qPCR analysis suggests that *Bm101* is not required for the onset of viral DNA replication in BmN cells.

The TCID₅₀ 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 h p.t. time points analyzed. As expected for vBm^{101-REP-PH-EGFP} and vBm^{PH-EGFP}-transfected cells, a strong increase in BV DNA was detected at 72 h p.t. In contrast, for vBm^{101-KO-PH-EGFP}-transfected cells no increase in BV DNA was detected above background at 72 h p.t. (Fig. 4b). The qPCR assay was therefore unable to detect any BV production by the *Bm101* knockout, in agreement with the TCID₅₀ results.

Discussion

Bm101 and its homologues are conserved in all lepidopteran NPVs and not found in dipteran and hymenopteran NPVs. *Bm101* consists of six transmembrane helices, which is conserved in all homologues. *Bm101* was found in BV and located in the envelope fraction of BV. However, the biological function of *Bm101* remains mainly uncharacterized due to the inability to construct a *Bm101* knockout virus. In this study, we constructed a *Bm101* knockout bacmid using the λ Red system. Our results show that *Bm101* is essential for the production of infectious progeny virus.

Fig. 4 qPCR analysis of viral DNA replication and BV DNA in culture supernatants in transfected BmN cells. **a** BmN cells were transfected in triplicate with vBm¹⁰¹-KO-PH-EGFP⁻, vBm¹⁰¹-REP-PH-EGFP⁻ or vBm^{PH}-EGFP⁻-bacmid. At the designated time points, total cellular DNA was isolated from the transfected cells, digested with the restriction enzyme DpnI to eliminate input bacmid, and analyzed by qPCR. **b** qPCR analysis of BV DNA in culture supernatants of BmN cells transfected with vBm¹⁰¹-KO-PH-EGFP⁻, vBm¹⁰¹-REP-PH-EGFP⁻ or vBm^{PH}-EGFP⁻-bacmid. qPCR was used to quantify BV DNA after 24, 48 and 72 h, using the primers ORF104-F and ORF104-R

The function of *Bm101* in the context of a BmNPV infection in BmN cells was analyzed with the *Bm101*-deletion bacmid. The mutant virus could not be propagated after transfection (Fig. 2a, b), suggesting that BV production or viral infectivity were affected. To test this, BV production and TCID₅₀ endpoint dilution assays were performed. The analysis showed that the deletion of *Bm101* affected infectious BV production. Comparison of the levels of viral DNA accumulation via real-time PCR in vBm¹⁰¹-KO-PH-EGFP⁻, vBm¹⁰¹-REP-PH-EGFP⁻ and vBm^{PH}-EGFP⁻-transfected cells showed that viral DNA synthesis was unaffected by the deletion of

Bm101 in the first 24 h p.t.. The difference between vBm¹⁰¹-KO-PH-EGFP⁻ and vBm¹⁰¹-REP-PH-EGFP⁻/vBm^{PH}-EGFP⁻ at 48 h p.t. is presumed to be caused by the different infectivity of BVs produced by the three bacmids. Polyhedra were found in vBm¹⁰¹-KO-PH-EGFP⁻-infected cells (Fig. 2b), indicating that the expression of the polyhedrin gene and viral replication were unaffected.

A number of baculovirus genes have been reported to affect BV production but do not appear to impact viral DNA synthesis, including *gp64*, *f-like protein*, *gp41*, *exon0*, *pp31*, *ac66*, *vlf-1*, *38K*, *ac101* (BV/ODV-C42), *ac142*, *ac143* and *me53* (Dai et al. 2004; de Jong et al. 2009; Fang et al. 2007; Ke et al. 2008; McCarthy and Theilmann 2008; McCarthy et al. 2008; Monsma and Blissard 1995; Monsma et al. 1996; Olszewski and Miller 1997; Oomens and Blissard 1999; Pearson et al. 2000; Vanarsdall et al. 2006, 2004; Wu et al. 2006; Yamagishi et al. 2007). The deletion or mutation of *ac142*, *ac143*, *gp41*, *vlf-1*, *38K*, *ac101*, *gp64*, *f-protein* abolishes infectious BV production whereas the deletion of *pp31*, *exon0* and *me53* only results in a reduction of BV production by 100–1,000-fold. Three genes *vlf-1*, *38K*, and *ac101* have been shown to impair the assembly of nucleocapsids. Loss or reduced BV production in the absence of GP41, EXON0, AC66 or AC142, however, is suggested to be due to the compromised nucleocapsid transport from the nucleus to the cytoplasm (Ke et al. 2008; McCarthy et al. 2008; Olszewski and Miller 1997). Loss of GP64 or F-protein affects the budding of nucleocapsids directly at the plasma membrane (Monsma et al. 1996; Oomens and Blissard 1999; Westenberg and Vlak 2008). In our previous study, we found that *Bm101* is the envelope protein of BV. It is likely that the assembly of BV is severely impaired in the absence of *Bm101*. *Bm101* in comparison to the above proteins therefore appears to be an important factor for the BV production. However the precise nature of the *Bm101* role in BV assembly and production, however remains to be determined.

Acknowledgments This research was supported by the National Natural Science Foundation of China (31100118, 31370790), Natural Science Foundation of Jiangsu Province (BK2011495), Natural Science Foundation of the Higher Education Institutions of Jiangsu Province (09KJB180001) and Startup Scientific Research Fund from Jiangsu University for Advanced Professionals (09JDG006).

References

- Blissard GW, Wenz JR (1992) Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. *J Virol* 66:6829–6835
- Chen H, Li M, Huang G, Mai W, Chen K, Zhou Y (2014) *Bombyx mori* nucleopolyhedrovirus ORF101 encodes a budded virus envelope associated protein. *Curr Microbiol* 69:158–163
- Dai X, Stewart TM, Pathakamuri JA, Li Q, Theilmann DA (2004) *Autographa californica* multiple nucleopolyhedrovirus exon0 (orf141), which encodes a RING finger protein, is required for efficient production of budded virus. *J Virol* 78:9633–9644
- de Jong J, Arif BM, Theilmann DA, Krell PJ (2009) *Autographa californica* multiple nucleopolyhedrovirus me53 (ac140) is a nonessential gene required for efficient budded-virus production. *J Virol* 83:7440–7448
- Fang M, Dai X, Theilmann DA (2007) *Autographa californica* multiple nucleopolyhedrovirus EXON0 (ORF141) is required for efficient egress of nucleocapsids from the nucleus. *J Virol* 81:9859–9869
- Granados RR, Lawler KA (1981) In vivo pathway of *Autographa californica* baculovirus invasion and infection. *Virology* 108:297–308
- Hefferon KL, Oomens AG, Monsma SA, Finnerty CM, Blissard GW (1999) Host cell receptor binding by baculovirus GP64 and kinetics of virion entry. *Virology* 258:455–468
- Herniou EA, Olszewski JA, Cory JS, O'Reilly DR (2003) The genome sequence and evolution of baculoviruses. *Annu Rev Entomol* 48:211–234
- Ke J, Wang J, Deng R, Wang X (2008) *Autographa californica* multiple nucleopolyhedrovirus ac66 is required for the efficient egress of nucleocapsids from the nucleus, general synthesis of preoccluded virions and occlusion body formation. *Virology* 374:421–431
- Keddie BA, Aponte GW, Volkman LE (1989) The pathway of infection of *Autographa californica* nuclear polyhedrosis virus in an insect host. *Science* 243:1728–1730
- Luckow VA, Lee SC, Barry GF, Olins PO (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J Virol* 67:4566–4579
- Lung OY, Cruz-Alvarez M, Blissard GW (2003) Ac23, an envelope fusion protein homolog in the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus, is a viral pathogenicity factor. *J Virol* 77:328–339
- McCarthy CB, Theilmann DA (2008) AcMNPV ac143 (odv-e18) is essential for mediating budded virus production and is the 30th baculovirus core gene. *Virology* 375:277–291
- McCarthy CB, Dai X, Donly C, Theilmann DA (2008) *Autographa californica* multiple nucleopolyhedrovirus ac142, a core gene that is essential for BV production and ODV envelopment. *Virology* 372:325–339
- Monsma SA, Blissard GW (1995) Identification of a membrane fusion domain and an oligomerization domain in the baculovirus GP64 envelope fusion protein. *J Virol* 69:2583–2595
- Monsma SA, Oomens AG, Blissard GW (1996) The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. *J Virol* 70:4607–4616
- O'Reilly DR, Lk Miller, Luckow VE (1992) Baculovirus expression vectors: a laboratory manual. Freeman, New York
- Olszewski J, Miller LK (1997) A role for baculovirus GP41 in budded virus production. *Virology* 233:292–301
- Oomens AG, Blissard GW (1999) Requirement for GP64 to drive efficient budding of *Autographa californica* multicapsid nucleopolyhedrovirus. *Virology* 254:297–314
- Pearson MN, Groten C, Rohrmann GF (2000) Identification of the lymantria dispar nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the Baculoviridae. *J Virol* 74:6126–6131
- Pijlman GP, Dortmans JC, Vermeesch AM, Yang K, Martens DE, Goldbach RW, Vlak JM (2002) Pivotal role of the non-hr origin of DNA replication in the genesis of defective interfering baculoviruses. *J Virol* 76:5605–5611
- Thiem SM, Miller LK (1989) Identification, sequence, and transcriptional mapping of the major capsid protein gene of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *J Virol* 63:2008–2018
- Vanarsdall AL, Okano K, Rohrmann GF (2004) Characterization of a baculovirus with a deletion of vlf-1. *Virology* 326:191–201
- Vanarsdall AL, Okano K, Rohrmann GF (2006) Characterization of the role of very late expression factor 1 in baculovirus capsid structure and DNA processing. *J Virol* 80:1724–1733
- Wang M, Tan Y, Yin F, Deng F, Vlak JM, Hu Z, Wang H (2008) The F-like protein Ac23 enhances the infectivity of the budded virus of gp64-null *Autographa californica* multi-nucleocapsid nucleopolyhedrovirus pseudotyped with baculovirus envelope fusion protein F. *J Virol* 82:9800–9804
- Wang M, Tuladhar E, Shen S, Wang H, van Oers MM, Vlak JM, Westenberg M (2010) Specificity of baculovirus P6.9 basic DNA-binding proteins and critical role of the C terminus in virion formation. *J Virol* 84:8821–8828
- Westenberg M, Vlak JM (2008) GP64 of group I nucleopolyhedroviruses cannot readily rescue infectivity of group II f-null nucleopolyhedroviruses. *J Gen Virol* 89:424–431
- Wu W, Lin T, Pan L, Yu M, Li Z, Pang Y, Yang K (2006) *Autographa californica* multiple nucleopolyhedrovirus nucleocapsid assembly is interrupted upon deletion of the 38K gene. *J Virol* 80:11475–11485
- Yamagishi J, Burnett ED, Harwood SH, Blissard GW (2007) The AcMNPV pp31 gene is not essential for productive AcMNPV replication or late gene transcription but appears to increase levels of most viral transcripts. *Virology* 365:34–47