Bombyx mori nucleopolyhedrovirus ORF56 encodes an occlusion-derived virus protein and is not essential for budded virus production

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Received 1 December 2007
Accepted 15 January 2008

Bombyx mori nucleopolyhedrovirus ORF56 (*Bm56*) is a baculovirus core gene that is highly conserved in all baculoviruses that have had their genomes sequenced to date. Its transcripts in BmNPV-infected cells could be detected from 12 h post-infection (p.i.) and the encoded protein could be detected at 16 h p.i. by using a polyclonal antibody against glutathione *S*-transferase–Bm56 fusion protein. Western blot analysis showed that Bm56 is a structural component of the occlusion-derived virus nucleocapsid. Subsequent confocal microscopy revealed that Bm56 was distributed in the outer nuclear membrane and the intranuclear region of infected cells. To investigate the role of Bm56 in virus replication, a *Bm56*-knockout bacmid of BmNPV was constructed via homologous recombination in *Escherichia coli*. The *Bm56* deletion had no effect on budded virus (BV) production in cultured cells; however, the deletion affected occlusion-body morphogenesis. A larval bioassay demonstrated that the *Bm56* deletion did not reduce infectivity, whereas it resulted in a 50 % lethal time that was 16–18 h longer than that of the wild-type bacmid at every dose used in this study. These results indicate that Bm56 facilitates efficient virus production *in vivo*; however, it is not essential for BV production *in vitro*.

INTRODUCTION

The *Baculoviridae* is a diverse family of pathogens that are infectious for arthropods, particularly insects of the order Lepidoptera. Members of the genus *Nucleopolyhedrovirus* (NPVs), a genus in the family *Baculoviridae*, typically produce two virion phenotypes of progeny virus: occlusion-derived virus (ODV) and budded virus (BV). The ODV transmits infection from insect to insect by infecting midgut columnar epithelial cells, whereas the BV is responsible for causing systemic infection within the host (Keddie *et al.*, 1989). The two viral forms are essential for natural propagation of baculoviruses.

Open reading frame 56 (ORF56 or *Bm56*) of *Bombyx mori* nucleopolyhedrovirus (BmNPV) is considered to be a core gene (Herniou *et al.*, 2003), with homologues existing in all baculoviruses that have had their genomes sequenced to date, including lepidopteran NPVs, lepidopteran granuloviruses, hymenopteran NPVs and a dipteran baculovirus. It was reported that *Culex nigripalpus* nucleopolyhedrovirus (CuniNPV) ORF58, which is homologous with *Bm56*, encodes a structural protein for ODV, as determined by the nano-electrospray quadrupole time-of-flight mass spectrometry (GeLC-MS/MS) method (Perera *et al.*, 2007). These data led to the proposal that *Bm56* probably plays a defined role in viral replication. However, knowledge about this common gene is still very limited.

In this study, we showed that *Bm56* is transcribed at 12 h post-infection (p.i.) and that its encoded protein could be detected at 16 h p.i. with polyclonal serum against glutathione *S*-transferase (GST)–Bm56. Confocal microscopy demonstrated that Bm56 was mainly distributed in the outer nuclear membrane and intranuclear region in BmNPV-infected cells. Western blot analysis showed that Bm56 is a structural component of the ODV nucleocapsid. Moreover, we utilized a bacmid of BmNPV that replicates in *Escherichia coli* to delete *Bm56* and evaluated the replication of the deletion bacmid in cultured cell lines. There was no difference observed between the *Bm56* deletion bacmid and BmNPV bacmid with respect to BV production. Additionally, in an *in vivo* assay, no significant difference in the 50 % lethal dose (LD_{50}) was observed between the *Bm56*-deleted bacmid and the wild type. However, the 50 % lethal time (LT_{50}) of the *Bm56*-deleted bacmid was 16–18 h longer than that of the wild-type

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A supplementary table showing oligonucleotide PCR primers that were designed for and used in this study is available with the online version of this paper.

BmNPV bacmid in this study. Thus, our data suggested that Bm56 is a structural component of ODVs that facilitates efficient virus production *in vivo*. However, Bm56 is not essential for BV production *in vitro*.

METHODS

Cells and viruses. BmNPV (ZJ strain) was propagated in BmN (BmN-4) cells maintained at 27 °C in TC-100 insect medium supplemented with 10 % (v/v) fetal bovine serum (Gibco-BRL). Virus titration and other routine manipulations were performed according to standard protocols (O'Reilly *et al.*, 1992).

Bacterial strains, bacmid DNA and plasmids. *E. coli* strain DH10B and pFastBac1 were purchased from Invitrogen. *E. coli* strain BW25113 (pKD46) was kindly provided by Dr Mary Berlyn (Yale University, New Haven, CT, USA); plasmid pKD46 contains the phage λ Red system under the control of an arabinose promoter. *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 kindly provided by Dr Enoch Y. Park (Shizuoka University, Shizuoka, Japan). The pRADZ3 plasmid, containing the chloramphenicol-resistance gene (Cm^R), was kindly provided by Dr Hua Yuejin (Zhejiang University, Zhejiang Province, China). All strains were cultured in Luria–Bertani (LB) medium with appropriate antibiotics. Plasmid pFastBacGFP (Wu *et al.*, 2006), containing the green fluorescence protein gene (*gfp*) under the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) *ie-1* promoter, was kindly provided by Dr Pang Yi (Sun Yat-sen University, Guangdong Province, China).

Expression of Bm56 and preparation of antibody. The *Bm56* coding region was amplified from BmNPV genomic DNA by PCR with primers Bm56F and Bm56R (see Supplementary Table S1, available in JGV Online), which were synthesized based on the genomic sequence of BmNPV T3 (GenBank accession no. NC_001962). *Bm56* was subcloned into the expression vector pGEX4t-2 with GST at the N terminus. Fusion protein GST–Bm56 was expressed in *E. coli* under induction by 0.1 mM IPTG at 37 °C, and retrieved after SDS-PAGE. Anti-GST–Bm56 serum was prepared by using standard techniques (Harlow & Lane, 1988). Purified GST–Bm56 protein (about 2 mg) in complete Freund's adjuvant was injected subcutaneously to immunize New Zealand white rabbits, followed by two booster injections in incomplete Freund's adjuvant, with a gap of 2 weeks before exsanguinations. The polyclonal rabbit antibody against GST–Bm56 was used for immunoassay.

RT-PCR analysis. For RT-PCR analysis, total RNA was extracted from mock- or BmNPV-infected cells at various time intervals (3, 6, 12, 16, 24, 48 and 72 h p.i.). Total RNA was purified by incubating with DNase I (Worthington Biochemical) to remove potential genomic DNA contamination. Purified RNA was examined by PCR with primers Bm56F and Bm56R (see Supplementary Table S1, available in JGV Online). RT-PCR was performed by using a RevertAid First Strand cDNA Synthesis kit (Fermentas) with 1 μg purified RNA as the template. First-strand cDNA was synthesized with avian myeloblastosis virus reverse transcriptase and oligo-$p(dT)_{18}$ primer. Subsequently, a nested PCR product was amplified with primers Bm56F and Bm56R (see Supplementary Table S1). PCR products were analysed on a 1.0 % agarose gel.

Temporal expression of Bm56 in infected BmN cells. For time-course analysis, 1×10^6 BmN cells were infected with BmNPV at an m.o.i. of 10. Cells were harvested at the designated times (4, 8, 12, 16, 24, 48 and 72 h) and washed with 1 × PBS (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) three times. The protein concentration of the cell extracts was determined by Bradford's method (Bradford, 1976). Cell lysates (20 μg) were analysed by SDS-PAGE (10 % gel) and subsequently subjected to Western blot assay.

Immunodetection of the Bm56 protein in ODVs and BVs. Preparation of ODVs and BVs and the fractionation of ODVs into envelope and nucleocapsids were performed as described previously (Xu *et al.*, 2006). The purified ODVs and BVs and the ODV nucleocapsid and envelope preparations were used for Western blot assay.

Immunofluorescence microscopy. BmN cells were infected with BmNPV at an m.o.i. of 5 and collected at 48 h p.i. The harvested cells were rinsed three times with 1 × PBS and fixed in cold methanol : acetone (1 : 1) for 15 min, followed by three washes with 1 × PBS. To detect the localization of Bm56, cells were incubated with anti-GST–Bm56 polyclonal antibody (1 : 400 dilution) in 1 × PBS for 2 h at room temperature. Primary antibody was removed by washing three times with 1 × PBS. The cells were incubated with protein G fused to EGFP for 2 h and the nucleus (DNA)-specific DAPI stain (Sigma) for 1 h. Subsequently, the cells were observed and photographed under a Zeiss LSM 510 confocal laser-scanning microscope.

Preparation of a linear fragment for homologous recombination. To generate *Bm56*-knockout virus by recombination in *E. coli* (Fig. 1a), we constructed a transfer vector (pET-ufs/Cm^R/dfs) in which the Cm^R gene was introduced to disrupt the *Bm56* coding region (corresponding to nt 54277–54280), and 219 bp of the 5′ end and 182 bp of the 3′ end were retained so that the deletion would not affect transcription of the adjacent genes (*lef-3* and *orf57*). Briefly, the transfer vector was constructed as follows. First, a 750 bp (nt 53527–54276) upstream flanking sequence (ufs) was PCR-amplified from BmNPV bacmid genomic DNA with primers De56UF and De56UR (see Supplementary Table S1, available in JGV Online) and cloned into pET-2 to generate pET-ufs. Second, a 1009 bp (nt 54281–55289) downstream flanking sequence (dfs) was amplified with primers De56DF and De56DR (see Supplementary Table S1) and cloned into pET-ufs to generate pET-ufs/dfs. Eventually, using the pRADZ3 plasmid as template, a 948 bp Cm^R sequence was amplified with primers Cm^RF and Cm^RR (see Supplementary Table S1) and cloned into pET-ufs/dfs to generate pET-ufs/Cm^R/dfs. The reconstructed vector was verified by sequencing.

The pET-ufs/Cm^R/dfs vector was then cleaved with restriction enzymes *Bam*HI and *Xho*I to generate a linear donor fragment (ufs/Cm^R/dfs). The linear donor fragment was used to electrotransform competent cells.

Generation of the *Bm56*-deleted bacmid. BW25113/pKD46 competent cells were made according to the method described by Datsenko & Wanner (2000). The BmNPV bacmid DNA was electrotransformed into BW25113/pKD46 competent cells to generate bacterial strain BW25113 containing pKD46 and BmNPV bacmid, designated BW25113/pKD46/BmBac.

Red system-induced BW25113/pKD46/BmBac electrocompetent cells were made as described by Pijlman *et al.* (2002). Briefly, the ufs/Cm^R/dfs fragment (100 ng) was mixed with 40 μl competent cells on ice. Electroporation was then performed by use of a Bio-Rad Gene Pulser II (2.5 kV, 25 Ω and 25 μF) and a 2 mm diameter cuvette, according to the manufacturer's instructions. Next, these cells were mixed with 800 μl pre-heated SOC medium (Sambrook & Russell, 2001) and incubated for 4 h at 30 °C with gentle shaking. The cells were collected and spread onto LB plates with kanamycin (50 μg ml^{-1}) and

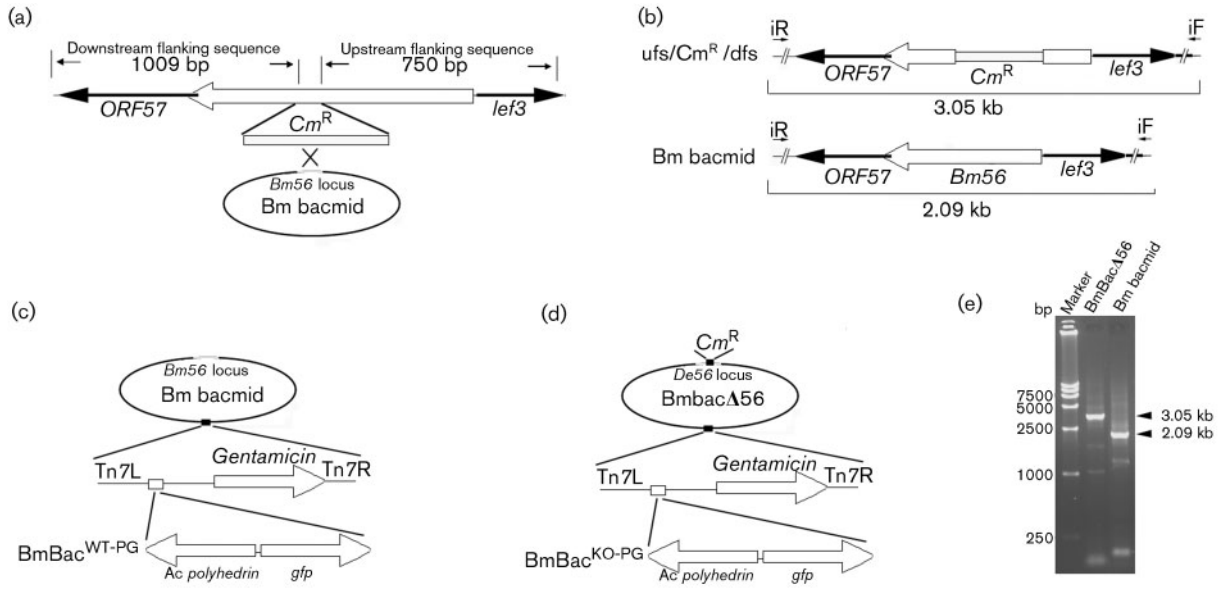

Fig. 1. Construction and PCR identification of BmBac^{KO-PG} and BmBac^{WT-PG}. (a) Scheme for construction of a *Bm56*-knockout BmNPV bacmid. The ORF of the *Bm56* locus was replaced by the chloramphenicol-resistance gene (*Cm*^{R}) via homologous recombination. (b) Scheme for identification of a *Bm56*-knockout bacmid and BmNPV bacmid with primers iF and iR. (c) Schematic diagram of BmBac^{WT-PG}, constructed by inserting *polyhedrin* and *gfp* into the BmNPV bacmid *polyhedrin* locus by Tn*7*-mediated transposition in the Bac-to-Bac system (Invitrogen). (d) Schematic diagram of BmBac^{KO-PG}, constructed by inserting the *polyhedrin* and *gfp* genes into the BmBacΔ56 bacmid *polyhedrin* locus. (e) PCR identification of the *Bm56*-deleted bacmid, BmBac^{KO-PG}. Sizes of PCR products are indicated.

chloramphenicol (7 μg ml^{-1}) for another 48 h. Finally, recombinant bacmid DNA was extracted and identified by PCR with primers iF and iR (see Supplementary Table S1, available in JGV Online). The identified BmNPV bacmid with the *Bm9* deletion was temporarily named BmBacΔ56 (Fig. 1d).

BmBacΔ56 DNA was extracted and electrotransformed into *E. coli* strain DH10B, designated DH10B/BmBacΔ56. Then, the helper plasmid (pMON7124) was chemically transformed into DH10B/BmBacΔ56 to generate DH10B cells containing the *Bm56*-deleted bacmid and the helper plasmid, designated DH10B/BmBacΔ56/helper, and subsequently used for marker-gene insertion.

Construction of BmNPV bacmid and *Bm56*-deleted bacmid containing *gfp* and *polyhedrin*. To facilitate examination of virus infection, we introduced donor plasmid pFB1-PH-GFP, which was generated by inserting the *polyhedrin* and *gfp* genes into pFastBac1 plasmid under the control of the *polyhedrin* promoter and the AcMNPV *ie-1* promoter, respectively (Wu *et al.*, 2006), by Tn*7*-mediated transposition in the Bac-to-Bac system (Invitrogen). pFB1-PH-GFP was transformed into BmDH10B and DH10B/BmBacΔ56/helper competent cells to generate BmBac^{WT-PG} (Fig. 1c) and BmBac^{KO-PG} (Fig. 1d), respectively. Successful transposition was verified by PCR with pUC/M13 forward and reverse primers.

BV growth curve. To determine the BV growth curve, BmN cells were infected with BmBac^{WT-PG} and BmBac^{KO-PG} at an m.o.i. of 5, then the supernatant was harvested at various times p.i. (8, 12, 16, 24, 48, 72, 96 and 120 h). BV titration was performed using an end-point dilution assay ($TCID_{50}$) (O'Reilly *et al.*, 1992).

Electron microscopy. The BmN cell monolayer was infected with BmBac^{KO-PG} at an m.o.i. of 5. At 96 h p.i., cells were harvested and the pellet was fixed in 2.5 % glutaraldehyde for 1 h at 4 °C, followed by fixation with 1 % osmium tetroxide for 1 h at room temperature. After the fixed cells were dehydrated in graded ethanol (50–100 %) and then soaked in acetone for 20 min, infiltration in graded Spurr resin (50–100 %) (Sigma) and incubation for 16 h at 70 °C were performed. After staining with uranyl acetate and lead citrate, ultrathin sections were viewed under a JEM-1230 transmission electron microscope (JEOL) at an accelerating voltage of 80 kV.

***B. mori* larval bioassay.** LD_{50} and LT_{50} values of BVs were determined by injection into the haemocoel of *B. mori* larvae, within 8 h of moulting to the fifth instar, of different doses of BVs (50, 500, 5000 and 50 000 p.f.u.) diluted in PBS. Twenty-five larvae per dose were used and each dose was repeated in triplicate. Mortality was determined every 4 h.

LT_{50} and LD_{50} values were estimated by using the DPS data processing system for practical statistics (Tang & Feng, 2002). Probit analysis (Finney, 1971) was adopted in the statistics.

RESULTS

Sequence and transcriptional analysis

The ORF of the *Bm56* gene is 405 nt (nt 54058–54462) in length and encodes a 134 aa peptide with a predicted molecular mass of about 15.8 kDa. A baculovirus consensus late transcriptional start motif, ATAAG, is found 68 nt upstream of the start codon, suggesting that *Bm56* might be a late-transcribed gene. Computer analysis showed that a

transmembrane region (aa 94–116) was predicted confidently at the C terminus. Transcriptional analysis by RT-PCR revealed that a PCR product with predicted size of 417 bp was detectable at 12 h p.i., and was still stable at the very late phase (72 h p.i.) (Fig. 2a). Thus, these results, coupled with the late consensus initiation sequence (ATAAG), indicate that *Bm56* is a late-transcribed gene.

Temporal expression of Bm56 in infected cells

To determine the time course of Bm56 protein expression, infected cells were harvested at designated time points, then analysed by Western blotting using anti-GST–Bm56 serum. The results revealed that a band with an apparent molecular mass of 42 kDa presented a strong antiserum reaction (Fig. 2b). This band was detectable as early as 16 h p.i., increased to high levels at 24 h p.i. and lasted until 72 h p.i. (Fig. 2b). However, the molecular mass of the detected protein (42 kDa) was larger than the predicted putative *Bm56* gene product (15.8 kDa). This might be due to post-translational modifications, a protein complex or some other processing functional form.

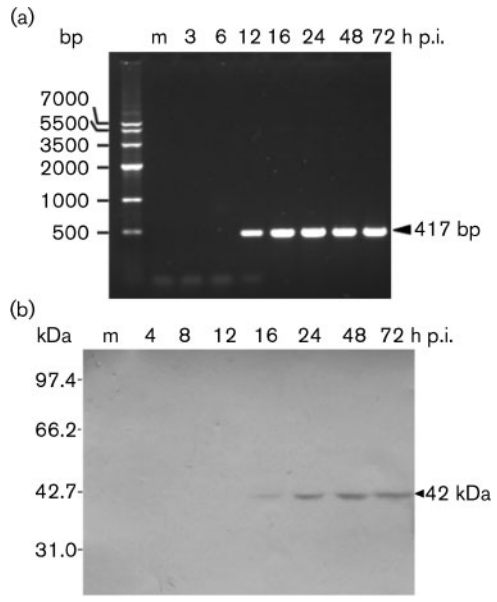

Fig. 2. Time course of Bm56 expression in infected BmN cells. (a) Transcriptional analysis by RT-PCR. Total RNA from mock-infected (m) or BmNPV-infected cells was extracted at the designated times p.i. (3, 6, 12, 16, 24, 48 and 72 h) and then treated with DNase I. cDNA was synthesized with oligo-$dT_{(18)}$ primer and PCR was performed with primers Bm56F and Bm56R. (b) Western blot analysis of Bm56 in infected BmN cells. Cells were collected at 0 (mock; m), 3, 6, 12, 24, 48 and 72 h p.i., and 20 μg cell lysate at each interval was subjected to Western blot analysis using anti-GST–Bm56 serum. Binding was detected with diaminobenzidine (DAB) as a chromogenic substrate. Protein markers are indicated on the left. The sizes of reactive bands are indicated by arrows.

Immunodetection of the Bm56 protein in ODVs

To determine whether the Bm56 protein is a structural component of BmNPV, Western blot analysis for purified BVs and ODVs was performed. The ODV fraction showed a reactive band of 42 kDa (Fig. 3a) and the size was in agreement with that detected in lysates from infected cells. In contrast, no band was detected in the BV fraction (Fig. 3a). Thus, the Bm56 protein appeared to be a structural protein specific for ODVs. To locate Bm56 precisely within ODVs, the ODV fraction was further separated into ODV nucleocapsid protein (ODV-NC) and ODV envelope protein (ODV-E) fractions. When

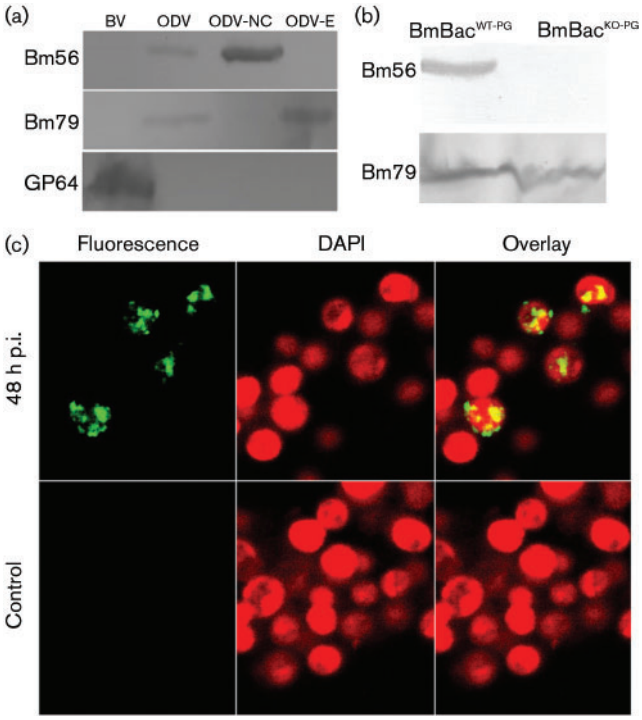

Fig. 3. Structural and subcellular localization of Bm56. (a) Western blot analysis of ODVs and BVs of BmNPV. Preparations of ODVs, ODV nucleocapsid (ODV-NC), ODV envelope (ODV-E) and purified BVs were subjected to Western blot analysis using anti-GST–Bm56, anti-GST–Bm79 (ODV-E28) or anti-GP64 serum. The primary antibodies used in the experiments are indicated to the left of each panel. (b) Western blot analysis of BmN cells infected with BmBac^{WT-PG} and BmBac^{KO-PG}. To confirm the specificity of the antibody against Bm56, preparations of BmBac^{WT-PG}- or BmBac^{KO-PG}-infected BmN cells were subjected to Western blot analysis using anti-GST–Bm56 and anti-GST–Bm79 (ODV-E28) sera, respectively. (c) Intracellular localization of Bm56 in BmNPV-infected BmN cells. Cells were collected at 48 h p.i., washed with 1× PBS and reacted with anti-GST–Bm56 serum; fluorescence was developed by incubating with protein G fused to EGFP. For a control, pre-immune serum was used as the primary antibody. Nuclei were stained with DAPI (red). Samples were observed under a confocal laser-scanning microscope.

ODV-NC and ODV-E were analysed with the antiserum against GST–Bm56, Bm56 was found only in the ODV-NC fraction and not in the ODV-E fraction. The efficacy of the fractionation was examined by immunoassay with antibody against Bm79 (ODV-E28, an ODV envelope-specific protein) (Xu *et al.*, 2006) and GP64 (a BV-specific protein). The results showed that positive bands of 28 and 64 kDa were detected only in the ODV-E fraction and BV sample, respectively (Fig. 3a). Thus, the separation of ODV-NC, ODV-E and BV was considered to be pure. Hence, the above results suggest that the *Bm56* gene encodes a structural protein associated with the nucleocapsid of ODVs.

Subcellular localization of Bm56

The subcellular localization of Bm56 was investigated by immunofluorescence using a confocal laser-scanning microscope. At 48 h p.i., BmNPV-infected cells were collected for fluorescence examination. High levels of Bm56 were distributed in the outer nuclear membrane and the intranuclear region (Fig. 3c). In contrast, no fluorescence was detected in uninfected cells (Fig. 3c).

Construction of BmBac^{KO-PG} and BmBac^{WT-PG}

To delete the *Bm56* gene in the BmNPV bacmid via λ Red recombination, we constructed a linear donor fragment (ufs/Cm^R/dfs) containing the ufs, *Cm*^{R} and dfs (Fig. 1a). The donor fragment was electrotransformed into BW25113/pKD46/BmBac competent cells to produce the *Bm56*-knockout bacmid BmBacΔ56. To verify that the deletion was derived precisely from the *Bm56* locus in the BmNPV bacmid genome, PCR analysis was performed with two specific primers (iF and iR) located at the extending region of the ufs and dfs, respectively (Fig. 1b). As expected, PCR products of 3.05 and 2.09 kb were amplified from BmBacΔ56 and BmNPV bacmid genomic DNA, respectively. Thus, the PCR results apparently confirmed that *Bm56* was deleted successfully from the *Bm56* locus in BmNPV bacmid DNA.

To facilitate observation of viral infection, the donor plasmid pFB1-PH-GFP, containing AcMNPV *polyhedrin* under the control of the AcMNPV *polyhedrin* promoter and *gfp* under the control of the AcMNPV *ie-1* promoter, was transposed into the *polyhedrin* locus in the BmNPV bacmid and *Bm56*-deleted bacmid to produce BmBac^{WT-PG} (Fig. 2c) and BmBac^{KO-PG} (Fig. 2d), respectively. A PCR assay with pUC/M13 forward and reverse primers confirmed successful transposition.

Bacmid infection and BV growth curve

To determine the effect of *Bm56* deletion upon virus replication, BmN cells were transfected with BmBac^{KO-PG} and BmBac^{WT-PG}. GFP expression by BmN cells with viral propagation was examined. At 96 h post-transfection, fluorescence was observed from the majority of cells with both types of bacmid transfection and the supernatants were collected for pass-through infection. Subsequently, fluorescence was observed from the second infection, indicating that DNA replication was occurring normally. The supernatants were then collected and BV titres were determined by $TCID_{50}$ assay. We observed that BmBac^{KO-PG} achieved a titre equivalent to that for BmBac^{WT-PG}.

To assess the effect of the *Bm56* deletion on viral replication quantitatively, we generated viral growth curves for BmBac^{WT-PG} and BmBac^{KO-PG}. Cells infected with BmBac^{WT-PG} had growth kinetics similar to those of BmBac^{KO-PG}, both reaching 10^9 $TCID_{50}$ ml^{-1} at 120 h p.i. (Fig. 4). The above data demonstrated that *Bm56* was not essential for BV production in cultured cells.

Electron microscopic observation

To investigate the effect of *Bm56* deletion on ODV and occlusion-body formation, thin sections generated from BmBac^{KO-PG}-infected cells were examined by electron microscopy. BmBac^{KO-PG}-infected cells exhibited features of characteristic baculovirus infection, including an enlarged nucleus, the presence of an electron-dense virogenic stroma (Fig. 5a) and enveloped nucleocapsids (Fig. 5c). However, compared with wild-type bacmid-infected cells, many strip-like and *de novo* envelope-like structures, to which partial nucleocapsids were attaching, were observed in the nuclei of cells infected with the *Bm56*-deleted bacmid (Fig. 5b, c). Additionally, *Bm56* deletion aborted occlusion-body formation, and only a polyhedron-like structure not containing ODVs was observed (Fig. 5a, d). Hence, the above observations suggested strongly that deletion of *Bm56* influences occlusion-body morphogenesis.

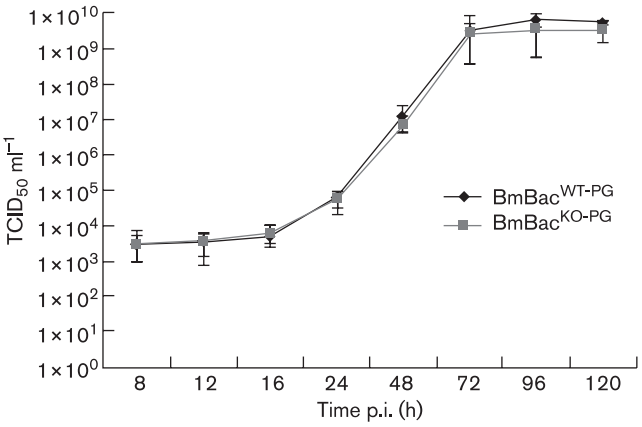

Fig. 4. Time-course analysis of BV production by one-step growth curve. BmN cells were infected at an m.o.i. of 5, and supernatant was collected at the designated times. The BV titre was determined by $TCID_{50}$. Error bars represent SD.

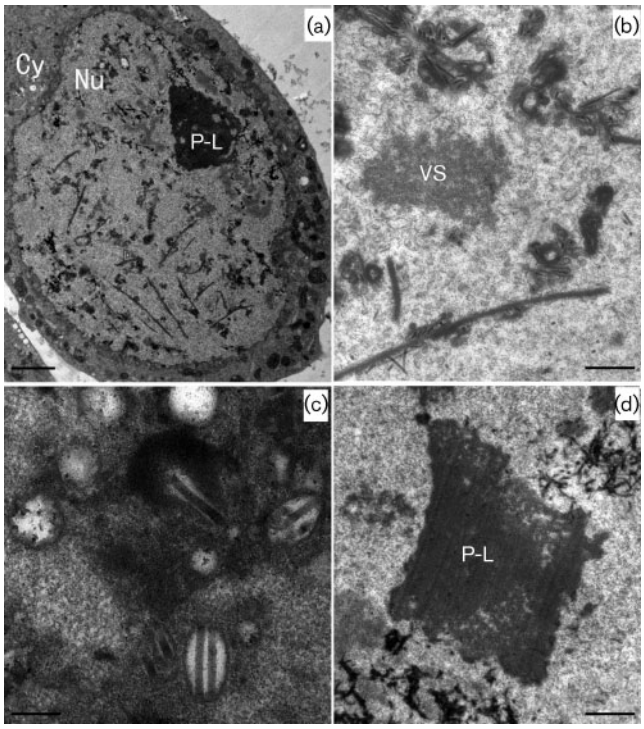

Fig. 5. Electron microscopic analysis of thin sections of BmN cells infected with the *Bm56*-deleted bacmid BmBac^{KO-PG}. (a) Image of a cell infected with BmBac^{KO-PG} at 4 days p.i., showing many *de novo* envelope-like and strip-like structures in the nucleus. (b) Higher magnification of a cell infected with BmBac^{KO-PG} at 4 days p.i., showing virogenic stroma in the nucleus and nucleocapsids gathered around the *de novo* envelope-like or strip-like structures. (c) Higher magnification of the enveloped nucleocapsids. (d) A polyhedron-like structure without embedded ODVs was observed. P-L, Polyhedron-like; Nu, nuclear; Cy, cytoplasm; VS, virogenic stroma. Bars, 2 μm (a); 1 μm (b, d); 0.2 μm (c).

Effect of *Bm56* deletion on BV infectivity in *B. mori* larvae

To determine whether the *Bm56* deletion has any effect on infectivity for *B. mori* larvae, fifth-instar larvae were injected in the haemocoel with BmBac^{KO-PG} and BmBac^{WT-PG} BVs. LD_{50} was determined by injecting with various doses of BVs (50, 500, 5000 and 50 000 p.f.u.). The data revealed that the LD_{50} values for BmBac^{KO-PG} and BmBac^{WT-PG} were 121.5 and 180.3 p.f.u. (Table 1), respectively. However, this difference was not statistically significant. Thus, the LD_{50} assay indicated that the *Bm56* deletion had no discernible effect on infectivity of BVs in *B. mori* larvae.

Table 1. Dose–mortality of BmBac^{WT-PG} and BmBac^{KO-PG} for fifth-instar *B. mori* larvae

Virus	LD_{50} (p.f.u.)	95 % confidence limits (p.f.u.)	
		Lower	Upper
BmBac^{WT-PG}	121.5	86.0	172.0
BmBac^{KO-PG}	180.3	113.8	285.8

Then, we examined the LT_{50} in fifth-instar *B. mori* larvae by injecting the larvae in the haemocoel with various doses of BV (50, 500, 5000 and 50 000 p.f.u.). We observed that the *Bm56*-deleted bacmid took about 16–18 h longer to kill *B. mori* larvae than the wild-type bacmid at every dose (Table 2). This observation suggested that the *Bm56* deletion reduced the efficiency of BV spreading *in vivo*.

DISCUSSION

Bm56 is a highly conserved gene; its homologues exist in all baculoviruses that have had their genomes sequenced to date, thus suggesting that Bm56 may perform important functions in the baculovirus life cycle. One of its counterparts, CuniNPV ORF58, was recently determined to be a structural protein for ODV by the GeLC-MS/MS method (Perera *et al.*, 2007). In this study, Bm56 was further determined by Western blot analysis to be located on the ODV nucleocapsid. There is general agreement that genes encoding structural proteins are transcribed at the very late phase, with products accumulating within the nuclei of virus-infected cells (Lu & Miller, 1997). Here, we observed that *Bm56* is transcribed at 12 h p.i. (Fig. 2a) and that its product (42 kDa) could be detected at 16 h p.i. (Fig. 2b), which indicated that *Bm56* is a late-transcribed gene. In our primary experiments, when a Western blot assay was performed to determine the expression profile of Bm56, we detected a 16 kDa protein in addition to one of 42 kDa (data not shown). However, the 16 kDa protein could not be confirmed by repeating the assay, although its molecular mass is similar to the theoretical size (15.8 kDa). To confirm the specificity of the antibody against Bm56, BmN cells infected with the *Bm56*-deleted virus (BmBac^{KO-PG}) were subjected to Western blot analysis. The results showed that no positive band was detected in BmBac^{KO-PG}-infected cells (Fig. 3b), which excluded the possibility that the anti-Bm56 serum recognized a non-related viral protein in addition to Bm56. A likely explanation for this observation is that the 42 kDa protein was due to some other processing functional form. A similar phenomenon was also observed in several other structural proteins, such as ODV-EC56 (Braunagel *et al.*, 1996a), ODV-EC43 (Fang *et al.*, 2003) and ODV-E18 (Braunagel *et al.*, 1996b). Additionally, confocal microscopy combined with immunoassay demonstrated that Bm56 localized to the outer nuclear membrane and the intranuclear region (Fig. 3c) where ODV formation occurred (Williams & Faulkner, 1997), assuming that Bm56 was transported from the cytoplasm into the nucleus after Bm56 synthesis. The property of transport has been reported in the ODV structural proteins ODV-E66 (Braunagel *et al.*, 2004) and ODV-E25 (Hong *et al.*, 1997). It has not been determined whether Bm56 shares this phenomenon.

With respect to BV production, no difference was observed between the *Bm56*-deleted bacmid and wild-type BmNPV

Table 2. Time–mortality of $BmBac^{WT\text{-}PG}$ and $BmBac^{KO\text{-}PG}$ for fifth-instar *B. mori* larvae

Virus/dose per larva (p.f.u.)	LT_{50} (h)	95 % confidence limits (h)	
		Lower	Upper
$BmBac^{WT\text{-}PG}$			
50 000	129.6	125.9	133.3
5000	135.3	131.8	138.8
500	141.0	136.5	145.5
50	–*		
$BmBac^{KO\text{-}PG}$			
50 000	147.0	141.4	152.6
5000	152.5	147.3	156.7
500	159.4	155.1	163.7
50	–*		

*Final mortality was <50 %.

bacmid (Fig. 4) *in vitro*. Also, an assay using larvae injected intrahaemocoelically with BV revealed that there was no statistically significant difference in LD_{50} between *Bm56*-deleted bacmid and BmNPV bacmid (Table 1). The above observations also applied to the gene encoding fibroblast growth factor (VFGF) in baculovirus (Detvisitsakun *et al.*, 2006). In contrast to *vfgf*-deleted bacmid, which did not have any effects on the LT_{50} in susceptible larvae infected intrahaemocoelically (Detvisitsakun *et al.*, 2007), *Bm56*-deleted bacmid had an LT_{50} that was 16–18 h longer than that for the wild-type BmNPV bacmid (Table 2). To examine the effects of *Bm56* deletion on ODV and polyhedron formation, cells were prepared for electron microscopic observation. The results demonstrated that Bm56 deletion has an effect on occlusion-body morphogenesis. Polyhedron-like structures were observed in the nucleus, and they were incapable of infecting silkworm larvae via the midgut (data not shown). In general, the above results suggest that Bm56 is a structural component of ODVs, but is not essential for BV production in cultured cells. However, Bm56 has advantageous effects on BV infectivity *in vivo*.

ACKNOWLEDGEMENTS

We thank Dr Boardman and Miss Oman (Indiana University School of Medicine, Indianapolis, IN, USA) for helpful reviewing of this paper. This project was supported by the National Program of High-Tech Research and Development (863 High-Tech Program, no. 2006AA10A119) and the National Natural Science Foundation of China (project no. 30570074).

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