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Bm65 is Essential for the Propagation of *Bombyx mori* Nucleopolyhedrovirus

Qi Tang · Guohui Li · Qin Yao · Liang Chen · Fan Feng · Yi Yuan · Keping Chen

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Abstract *Orf65* (*Bm65*) of *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) is a highly conserved gene that encodes an unknown 104-amino acid protein. In the present study, we have shown the role of Bm65 in the baculovirus life cycle. 5′-RACE analysis showed that the transcription start site of Bm65 was 14 nucleotides upstream of the start codon ATG. The transcription profile of Bm65 was detected from 6 to 72 h postinfection (p. i.) by RT-PCR. A Bm65-knockout bacmid was constructed by homologous recombination to characterize the role of *Bm65* in viral life cycle. Fluorescence microscopy showed that *Bm65*-knockout virus was unable to generate infectious budded virus in BmN cells. Furthermore, quantitative real-time PCR analysis demonstrated that *Bm65* deletion did not affect the viral DNA replication. To conclude, *Bm65* is essential for the propagation of *Bm*NPV, but is unnecessary for the replication of viral DNA.

Qi Tang and Guohui Li have contributed equally to this study.

Q. Tang
School of Food and Biological Engineering, Jiangsu University, 301# Xuefu Road, Zhenjiang 212013, China
e-mail: tangqi1224@163.com

Q. Tang · G. Li · Q. Yao · L. Chen · F. Feng · Y. Yuan · K. Chen (✉)
Institute of Life Sciences, Jiangsu University, 301# Xuefu Road, Zhenjiang 212013, China
e-mail: kpchen@ujs.edu.cn

Introduction

Baculoviruses are enveloped, double-stranded DNA viruses, which infect arthropods, primarily insects from the order Lepidoptera and also insects in the orders Hymenoptera and Diptera. During the baculoviruses life cycle, two progeny virion phenotypes are produced: Budded Virus (BV) and Occlusion-Derived Virus (ODV). These two virions possess similar nucleocapsid structures and identical genetic information, but differ in the envelope composition and play different roles in viral life cycle. BV is responsible for spreading infections among cultured cells and tissues in the host, and ODV is required for the oral infection [1, 2]. Up to now, the genomes of 57 baculovirus have been sequenced, and the genomes range in size from 80 to 180 kbp [3, 4].

Gene expression in baculovirus follows a temporal cascade, which is mainly regulated at the transcriptional level. According to their transcriptional phases, baculovirus genes can be subdivided into early, late and very late genes. It is generally accepted that early genes are transcribed by host RNA polymerase II, and the products of early genes regulate the expression of late gene and very late genes. BmNPV is one of the most extensively studied members of the family *Baculoviridae*, with a genome of 128 kbp, potentially containing 143 putative open reading frames (ORFs) [5]. To date, most genes in BmNPV genome have been functionally characterized, some of which are identified as early genes such as *orf9*, *orf41*, and *orf122* [6–8]. However, the function of *Bm65* remains unknown.

In this study, the transcription of *Bm65* in virus-infected BmN cells was analyzed. In addition, a *Bm65*-knockout mutant was generated by homologous recombination in *E. coli* to investigate the role of *Bm65* in the BmNPV life cycle. Our results indicate that *Bm65* is an early gene, and the Bm65 deletion virus fails to produce infectious BV.

Materials and Methods

Computer-Assisted Sequence Analysis

The functional motif of *Bm65* was predicted using softwares of the EBI server (http://www.ebi.ac.uk) and the ExPASy server (http://www.expasy.ch). A variety of protein sequences, homologous to Bm65, were loaded from the updated GenBank/EMBL databases. Multiple sequences alignment was performed with ClustalW and edited with Genedoc software.

Bacmid, Virus, and Cells

BmNPV genome was donated by Dr. Enoch Y (Park of the Department of Applied Biological Chemistry, Shizuoka University, Japan), which was propagated in *E. coli* strain DH10B, containing a BmNPV genome. *E. coli* strains DH5α, respectively, containing plasmid pBADgbaA and pKOV-Cm, were kindly donated by Dr. Kai Yang (State Key Laboratory of Biocontrol, Sun Yat-sen University). *E. coli* Rosetta 2 (DE3) pLysS strain was maintained in our laboratory. pFB-ieGP was constructed by Dr. Hongxing Shen in our laboratory [9]. BmN cells were cultured at 27 °C in TC-100 insect medium supplemented with 10 % fetal calf serum (Gibco).

Transcription Analysis of Bm65

Total RNA was extracted from 1.6×10^6 BmN cells mock-infected and infected with BmNPV at a multiplicity of infection (MOI) of 5 at 0, 6, 12, 24, 48, 72. and 96 h p. i., respectively, using the TRIZOL® Reagent from Invitrogen.

After mRNA was digested with RNase-free DNaseI (TaKaRa) to remove the viral genome contamination, first-strand cDNA synthesis was carried out with 3 μg mRNA as template per time point by means of ThermoScript™ RT-PCR system Kit (Invitrogen) according to the manufacturer's instructions. DNA fragment of *Bm65* was amplified by PCR using two primers: 65-F and 65-R. In addition, BmNPV *ie1* genes were used as the control for early gene with *ie1*-F and *ie1*-R, and *vp39* genes were used as the control for late gene with *vp39*-F and *vp39*-R. Total RNA from mock-infected cells was used as a negative control.

The 5′ end of *Bm65* transcripts was determined with the 5′ rapid amplification cDNA ends (RLM-RACE) kit (Ambion), using 3 μg extracted total RNA from 24 and 48 h p. i. as template. A random-primed reverse transcription reaction was performed to synthesize the first-strand cDNA with random decamers. The first PCR was performed with 5′-RACE outer primer and GSP1, and nested PCR was carried out to amplify the 5′ end of *Bm65* transcript with 5′-RACE inner primer and GSP2. These primer sets were listed in Table 1. The PCR products were gel purified and cloned into pMD18-T (TaKaRa), and sequenced with M13F(-47) or M13R(-48) primers.

Deletion of *Bm65*

A BmNPV bacmid with a deletion of *Bm65* was constructed in *E. coli* by ET recombination system as described previously by Li et al. [10]. We first constructed a transfer vector in which the *Bm65* locus region was replaced with Chloramphenicol (Cm) for antibiotic selection in *E. coli*. Primers 65US-F and 65US-R were used to amplify a 588-bp 5′ flank of *Bm65* from the BmNPV bacmid. The PCR product was digested with *Hin*dIII/*Pst*I and ligated into the pUC18 plasmid to generate the recombinant plasmid pUC18-US. Then, a 1,039-bp Cm gene cassette was amplified from pKOV-Cm with Cm-F and Cm-R. After being digested with *Bam*HI and *Pst*I, the PCR product was ligated into vector pUC18-US to generate the recombinant plasmid pUC18-US-Cm. Finally, a 653-bp 3′ flank of *Bm65* was amplified from the BmNPV bacmid with the primers 65 DS-F and 65DS-R. These primer sets were listed in Table 1. The resulting product was digested with *Bam*HI/*Kpn*I and ligated into the pUC18-US-Cm plasmid to generate a final *Bm65* knockout transfer vector named pUC18-US-Cm-DS. This transfer vector was digested with *Hin*dIII and *Kpn*I. Then, the linear 2,280-bp fragment containing the Cm gene cassette and the *Bm65* flanking region was gel purified and resuspended in distilled water.

A *Bm65* knockout BmNPV bacmid was generated by means of a modification of the λ Red recombinase system. Plasmid pBAD-gba which can provide λ Red recombination function was transformed into DH10B cells containing BmNPV bacmid. Then, the resulting clone cells were induced by 0.1 % L-arabinose to allow for the expression of λ Red system and electro-transformed with 1 μg purified linear 2,280-bp fragment. The electroporated cells were incubated at 37 °C for 1 h in 1 ml SOC medium, and then 100 μl of cell suspension was spread onto low salt agar medium containing 20 μg/ml chloramphenicol, 100 μg/ml ampicillin, and 50 μg/ml kanamycin. Plates were incubated at 37 °C for 2 days. Colonies resistant to chloramphenicol and kanamycin were selected for further research. The resulting *Bm65*-knockout bacmid was confirmed by PCR. The relative positions of the primers are shown in Fig. 3b. Cm-F and Cm-R were used to detect the correct insertion of the Cm gene cassette. Cm-F/65-R, Cm-F/65DS-R, 65-F/Cm-R, and 65US-F/Cm-R were used to examine the junction between the upstream or downstream flanking region and Cm. 65US-F and 65DS-R will amplify a 1,352-bp wild-type fragment and a 2,280-bp knockout fragment. Primer pairs, 65-F/65-R, 65-F/65DS-R, and 65US-F/65-R, were used to confirm the deletion of the *Bm65* gene and the correct insertion of the Cm gene cassette.

Table 1 Primers used for PCR procedure in the experiments

Primers	Sequence	PCR product length		
		Primer pairs	WT	KO
65-F	5′-ATGGATCCATGGCGACGACTCT-3′	(65-F/65-R)	328 bp	1,246 bp
65-R	5′-GCAAGCTTCAACTTATTTGCTAACAGA-3′			
iel-F	5′-AACGCGTCGTACACCAGT-3′	(Cm-F/Cm-R)	No product	1,039 bp
iel-R	5′-CCGTGCAAATGTTCGTGT-3′			
vp39-F	5′-GCCGCGACAA ATGAGAGT-3′	(Cm-F/65-R)	No product	1,171 bp
vp39-R	5′-GTTCGGGTT TGTGGTGTC-3′	(Cm-F/65DS-R)	No product	1,692 bp
5′-RACE outer primer	5′-GCTGATGGCGATGAATGAACACTG-3′			
GSP1	5′-GCAAGCTTCAACTTATTTGCTAACAGA-3′	(65-F/Cm-R)	No product	1,114 bp
5′-RACE inner primer	5′-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGAT	(65-F/65DS-R)		
GSP2	5′-GCTTTGATGAGACGCAATTTGAAAT-3′		839 bp	1,767 bp
65US-F	5′-ATAAGCTTCTCAAGC ACGCCACTCTGC-3′	(65US-F/Cm-R)	No product	1,627 bp
65US-R	5′-TACTGCAGTTTT CCATTGTCCTGCCC-3′			
Cm-F	5′-GGATCCCTTCGAATAAATACCTGTGA-3′	(65US-F/65-R)	831 bp	1,759 bp
Cm-R	5′-CTGCAGAACCAGCAATAGACATAAGC-3′			
65 DS-F	5′-TAGGATCCG CGAGCGCGTACGACT-3′	(65US-F/65DS-R)	1,352 bp	2,280 bp
65 DS-R	5′-AAGGTACCCA TGTACTTGCTCCACAGACTG-3′			
65REP-F	5′-CGGAATTCA GCATGTTCCAAAACGC-3′	(*ie1-F/ie1-R*)	535 bp	
65REP-R	5′-TACTGCAGCGGGCTCGCTAAATTGT-3′	(*vp39-F/vp39-R*)	472 bp	
Bm60352F	5′-CGTAGTGATAGTAATCG CCGC-3′			
Bm60452R	5′-AGTCGAGTCGCGTCGCTTT-3′			

Underlined letters indicate enzyme digestion site

WT wild type Bm-Bacmid, *KO* Bm-Bacmid with a deletion of *Bm65*

Construction of Knockout, Repair, and Wt BmNPV Bacmids Containing Polyhedrin and Green Fluorescent Protein Genes

To conveniently examine the effect of *Bm65* knockout on viral propagation and occlusion body (OB) morphogenesis, *polyhedrin* (*polh*) and green fluorescence protein (*gfp*) genes were introduced into the *polh* locus of BmNPV bacmid by transposition as previously described [11]. To construct a repair bacmid, a 646-bp DNA fragment containing *Bm65* gene with native promoter and polyadenylation signal was amplified with primers, 65REP-F and 65REP-R (Table 1). After being digested with *Eco*RI/*Pst*I, the PCR product was cloned into pFB-ieGP, which contained the *polh* gene with native promoter and polyadenylation signal from BmNPV and *gfp* gene (promoter from BmNPV *ie1*), to generate the pFB-ieGP-*Bm65*. Electrocompetent DH10B cells containing the pMON7124 helper plasmid and $\mathrm{Bm}^{\mathrm{Bm65KO}}$ were transformed with either the pFB-ieGP or pFB-ieGP-*Bm65* donor plasmid to generate the *Bm65*-knockout bacmid $\mathrm{Bm}^{\mathrm{Bm65KO\text{-}GP}}$ or the Bm65-repair bacmid $\mathrm{Bm}^{\mathrm{Bm65Rep\text{-}GP}}$.

At the same time, electrocompetent DH10B cells containing pMON7124 helper plasmid and BmNPV bacmid were transformed with pFB-ieGP to generate a control virus named $\mathrm{Bm}^{\mathrm{WT\text{-}GP}}$. After being incubated at 37 °C for 4 h in 1 ml SOC medium with moderate shaking, the transformed cells were spread onto LB agar medium containing 20 μg/ml chloramphenicol, 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml X-Gal, and 40 μg/ml IPTG plates, and then incubated at 37 °C for 48 h. The typical white colonies were selected and confirmed by PCR analysis.

Virus Growth Curve

To determine whether *Bm65* is required for viral propagation, a viral growth curve analysis was carried out as previously described [10]. In brief, BmN cells (10^6 cells/well) were transfected in triplicate with each bacmid DNA (2 μg/well) using Cellfectin Reagent (Invitrogen Life Technology), and the culture supernatants were collected at indicated time points. BV titers were determined using a TCID_{50} end-point dilution assay. Virus infection was determined by monitoring *gfp* expression through fluorescence microscopy. A well of the culture plate was identified as positive if there were one or more cells expressing GFP.

DNA Synthesis Analysis by Real-Time PCR

Quantitative real-time PCR was used to assess viral DNA replication as described previously [11]. A six-step standard calibration curve was generated with quadruplicate samples of purified bacmid DNA serially diluted from 50 to 0.0005 ng to quantify viral DNA replication in BmN cells. Then, BmN cells (10^6 cells/well) were transfected in quadruplicate with 2.0 μg of each bacmid ($Bm^{WT\text{-}GP}$, $Bm^{Bm65KO\text{-}GP}$, and $Bm^{Bm65Rep\text{-}GP}$), and cells were harvested at different time points. Total DNA was extracted from transfected BmN cells at 0, 6, 12, 18, 24, 48, and 96 h p.t., respectively, using Universal Genomic DNA Extraction kit (TaKaRa) according to manufacturer's instructions. To eliminate the interference of input bacmid DNA, the total DNA was digested with 10 units of *Dpn*I restriction enzyme (NEB) overnight in 50 μl total reaction volume. Quantitative PCR (qPCR) was performed with the digested DNA using the SYBR remix Ex Taq kit (TaKaRa) on the Mx3000P real-time PCR machine (Agilent Technologies). A 100-bp DNA fragment of BmNPV gene was amplified under the following conditions: 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, and 55 °C for 30 s with primers Bm60352F and Bm60452R (Table 1) as previously described [9].

Results

Sequence Analysis of Bm65

Bm65 locates at 59,670–59,984 nt in BmNPV genome and encodes a putative 104-amino-acid protein and the predicted molecular weight was ~12.2 kDa. A typical early promoter motif TATA is located −7 to−4 nt upstream of the start codon ATG. A polyadenylation signal (AATTT) was found +6 nt downstream of the stop codon TAA, which might function as the mRNA transcription termination and polyadenylation signal. No signal peptide was found in the sequence of Bm65 with the software in SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). In addition, no modification was predicted in the sequence of Bm65 by the softwares in ExPASy server and EBI server.

Bm65 is a highly conserved gene, and its homologs are found in most sequenced lepidopteran baculovirus genomes by BLAST (Basic Local Alignment Search Tool). However, its function is poorly understood. Alignment results showed that, among the homologs of Bm65, *Autographa californica* multiple nucleopolyhedrovirus *orf79* (Ac79), has the highest identity (99 %), while *Apocheima cinerarium* nucleopolyhedrovirus *orf74* (Apci74) has the lowest identity (34 %) with Bm65. In addition, BLAST searching showed that Bm65 is a member of GIY-YIG-like endonuclease superfamily (Fig. 1).

Analysis of Bm65 Transcripts

RT-PCR was performed to examine the temporal expression of *Bm65* transcripts using the total RNA extracted from BmN cells infected with BmNPV at different p. i.. *ie1* and *vp39* of BmNPV were used as controls for the early gene and late gene, respectively. The results revealed that a specific DNA fragment of *Bm65* about 328 bp was amplified from 6 to 72 h p. i., and the 535-bp *ie1* fragment was amplified from 3 to 72 h p. i., and the 472-bp *vp39* fragment was amplified from 12 to 96 h p.i (Fig. 2a). The amplified products were cloned into pMD18-T vector, and confirmed by DNA sequencing. No RT-PCR product was detected in mock-infected BmN cells.

Total RNAs were extracted from BmN cells infected with BmNPV at 6 h p. i. and 24 h p. i., and used to

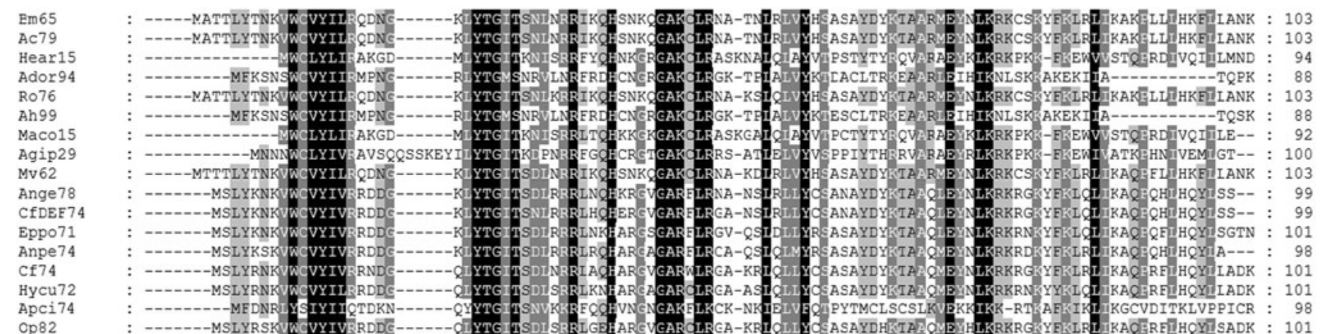

Fig. 1 Amino acid alignment of Bm65 and its homologous proteins. The alignment was performed using Clustal W and edited using Genedoc software. Identical amino acids are denoted by *black shading* and similar amino acids are denoted by *gray shading*. These sequences are from Genbank, and the accession numbers are as follows: BmNPV (YP_002884307.1), AcMNPV (NP_047482.1), HearMNPV (YP_002332551.1), AdorNPV (YP_002300612.1), RoMNPV (NP_703069.1), AhMNPV (NP_818746.1), MacoNPV (NP_613100.1), AgipNPV (YP_002268059.1), MvMNPV (YP_950792.1), AngeNPV (YP_803472.1), CfDEFMNPV (NP_932683.1), EppoNPV (NP_203240.1), AnpeNPV (YP_611042.1), CfMNPV (NP_848385.1), HycuNPV (YP_473260.1), ApciNPV (ADB84435.1), and OpMNPV (NP_046238.1)

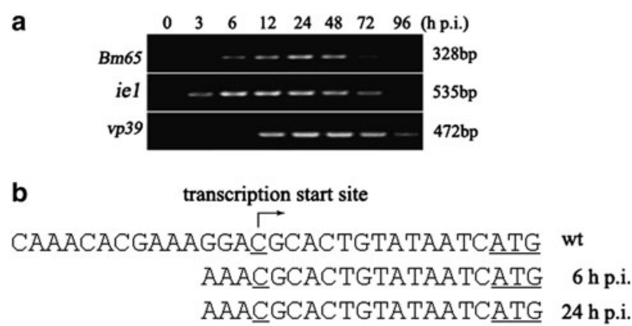

Fig. 2 Temporal expression of *Bm65* transcripts in BmNPV-infected BmN cells. **a** Transcription phase analysis of *Bm65* by RT-PCR. The genes are indicated on the *left*. *Numbers* above the lanes indicate times (h p. i.). The sizes (in bp) of different PCR products are indicated on the *right*. *ie1* and *vp39* are positive controls for early and late genes, respectively. **b** 5′-RACE analysis of the *Bm65* transcriptional start site. Alignment of the *Bm65* promoter with the sequences obtained from 6 and 24 h p. i. BmN cells. The translation start codon (ATG) is *underlined*. The *arrowhead* marks the transcription initiation site of *Bm65*

determine the transcriptional initiation site of the *Bm65* transcripts by 5′-RACE analysis. The result indicated that the transcription of *Bm65* is initiated at the site of 14 nt upstream of the ATG translation initiation codon (Fig. 2b).

Construction of Bm65-Knockout, Repair, and Wt BmNPV Bacmid

To investigate whether *Bm65* is required for viral propagation, *Bm65*-knockout, repair, and wild-type (wt) BmNPV bacmids containing *gfp* and *polh* were constructed.

In *Bm65* knockout bacmid ($Bm^{Bm65\text{-}KO}$), a 111-bp fragment inside the *Bm65* coding region (nt 59, 808–59, 918) was replaced with Cm by homologous recombination (Fig. 3a), which was confirmed by PCR analysis with different primer pairs (Fig. 3b, c). Primers CmF/CmR amplified a 1,039-bp fragment from $Bm^{Bm65\text{-}KO}$, but no PCR product from wt bacmid. Primers Cm-F/65-R amplified a 1,171-bp fragment from $Bm^{Bm65\text{-}KO}$, but no PCR product from wt bacmid. Primers Cm-F/65DS-R amplified a 1,692-bp fragment from $Bm^{Bm65\text{-}KO}$, but no PCR product from wt bacmid. Primers 65-F/Cm-R amplified a 1,114-bp fragment from $Bm^{Bm65\text{-}KO}$, but no PCR product from wt bacmid. Primers 65-F/65-R amplified a 1,246-bp fragment from $Bm^{Bm65\text{-}KO}$ and a 334-bp fragment from wt bacmid. Primers 65-F/65DS-R amplified a 1,767-bp fragment from $Bm^{Bm65\text{-}KO}$ and a 839-bp fragment from wt bacmid. Primers 65US-F/Cm-R amplified a 1,627-bp fragment from $Bm^{Bm65\text{-}KO}$, but no PCR product from wt bacmid. Primers 65US-F/65-R amplified a 1,759-bp fragment from $Bm^{Bm65\text{-}KO}$ and a 831-bp fragment from wt bacmid. Primers 65US-F/65DS-R amplified a 2,280-bp fragment from $Bm^{Bm91\text{-}KO}$ and a 1,352-bp fragment from wt bacmid.

To facilitate the observation of BV propagation and occlusion morphogenesis, *polh* and *gfp* were inserted into the polyhedron locus of *Bm65* knockout bacmid by Tn7 mediated transposition. The resulting bacmid was named $Bm^{Bm65KO\text{-}GP}$. $Bm^{WT\text{-}GP}$ as a positive control was constructed by transposing *polh* and *gfp* into the *polh* locus of BmNPV bacmid. In addition, $Bm^{Bm65Rep\text{-}GP}$ as a rescue control was constructed by transposing *Bm65* gene with its own promoter and poly(A) signal, as well as *polh* and *gfp* into the *polh* locus of BmNPV bacmid. All constructs were confirmed by PCR analysis.The accomplishment of the transposition events were also confirmed by the *gfp* expression and OB formation in the bacmid-transfected BmN cells.

Replication Analysis of Recombinant Bacmids in Transfected BmN Cells

To further assess whether *Bm65* is required for virus production, BmN cells were transfected with $Bm^{WT\text{-}GP}$, $Bm^{Bm65KO\text{-}GP}$ or $Bm^{Bm65Rep\text{-}GP}$ bacmid and observed by fluorescence microscopy. Fluorescence could be observed in the BmN cells transfected with $Bm^{WT\text{-}GP}$ and $Bm^{Bm65Rep\text{-}GP}$ bacmid DNA as early as 48 h p. t., and the fluorescent signal increased rapidly with time. However, at all time points, less than 10 % cells were observed expressing GFP in the BmN cells transfected with $Bm^{Bm65KO\text{-}GP}$ bacmid DNA (Fig. 4a). In addition, the transfected supernatants were collected and then used to infect BmN cells. The results demonstrated that fluorescence could be observed in almost 100 % BmN cells infected with the 48 h p. i. infectious supernatants from $Bm^{WT\text{-}GP}$ or $Bm^{Bm65Rep\text{-}GP}$ bacmid transfected BmN cells (data not shown). In contrast, in the cells incubated with the supernatant from $Bm^{Bm65KO\text{-}GP}$ bacmid-transfected BmN cells, no GFP expression was observed.

To further confirm the effect of *Bm65* on viral propagation, a virus growth curve was generated using the BV from bacmid-transfected BmN cells. The supernatants were collected at 24, 48, 72, or 96 h p.t., and the BV titers were determined by a $TCID_{50}$ endpoint dilution assay with BmN cells. The result revealed a significant difference ($P < 0.01$) on the slope of the growth curves between $Bm^{Bm65KO\text{-}GP}$ and $Bm^{WT\text{-}GP}$ or $Bm^{Bm65Rep\text{-}GP}$ (Fig. 4b).

Viral DNA Replication Analysis

To examine whether the deletion of *Bm65* affects the viral DNA replication, real-time PCR analysis was carried out to compare the initiation and viral DNA replication level among $Bm^{WT\text{-}GP}$-, $Bm^{Bm65Rep\text{-}GP}$-, and $Bm^{Bm65KO\text{-}GP}$ bacmid-transfected cells. We found similar DNA levels at 18 h p. t., but great differences ($P < 0.01$) between $Bm^{Bm65KO\text{-}GP}$

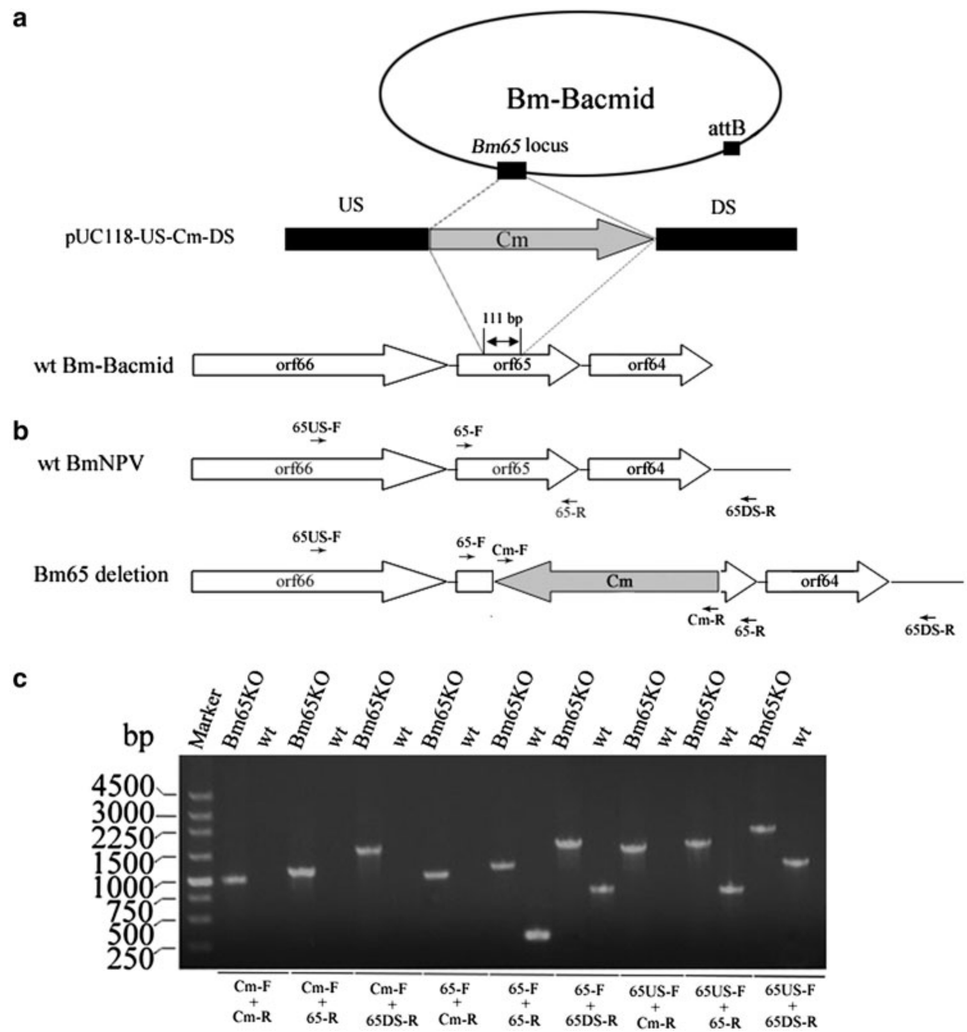

Fig. 3 Strategy for construction of *Bm65*-knockout BmNPV bacmid. **a** Schematic diagram showing the structure of *Bm65* locus in wild-type and *Bm65*-deleted virus, and the replacement of a 111-bp fragment with Cm resistance gene. **b** PCR primers for the *Bm65* gene deletion and insertion of Cm gene cassette. **c** PCR analysis with different primer pairs. Marker: DNA marker with sizes indicated. The virus templates are indicated above each lane, and the primer pairs used are shown *below*

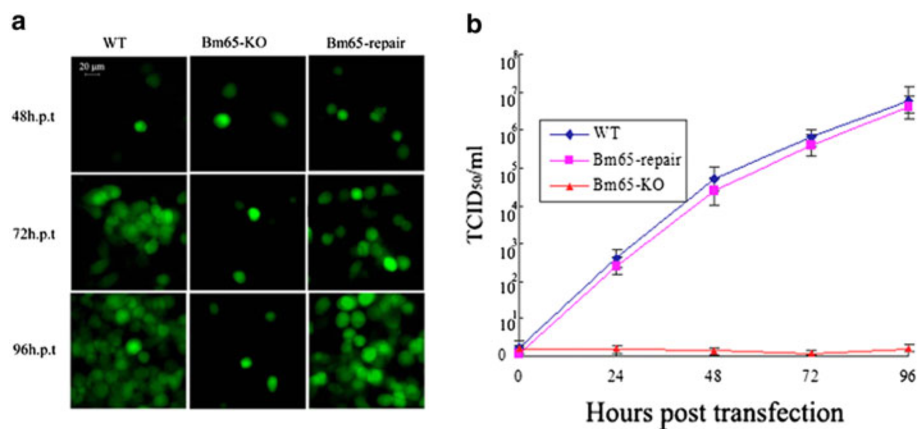

Fig. 4 Analysis of viral propagation in BmN cells. **a** Fluorescence microscopy of BmN cells transfected with $Bm^{WT\text{-}GP}$, $Bm^{Bm65KO\text{-}GP}$, or $Bm^{Bm65Rep\text{-}GP}$ at 48, 72, and 96 h p.t. **b** Virus growth curves generated from BmN cells transfected with $Bm^{WT\text{-}GP}$, $Bm^{Bm65KO\text{-}GP}$, or $Bm^{Bm65Rep\text{-}GP}$. Cells were transfected with 2.0 μg of bacmid DNA and cultured at 27 °C. Supernatants were harvested at the selected time points and assessed for infectious virus by $TCID_{50}$ assay. Each datum point represents the average titer derived from three independent transfected cultures. *Error bars* indicate standard deviations

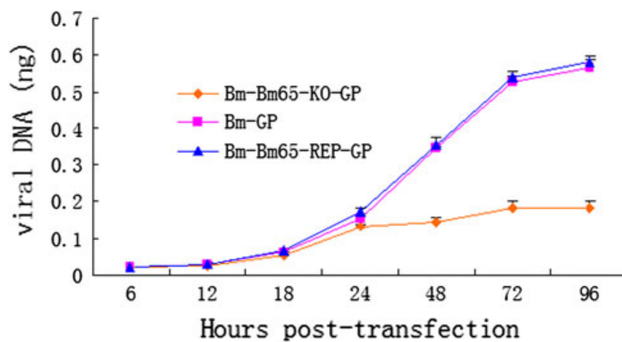

Fig. 5 Real-time PCR analysis of viral DNA synthesis. BmN cells were transfected in quadruplicate with Bm$^{\text{WT-GP}}$, Bm$^{\text{Bm65Rep-GP}}$, or Bm$^{\text{Bm65KO-GP}}$ bacmid. Total DNA was isolated at the designated time points, digested with the restriction enzyme DpnI to eliminate input bacmid, and analyzed by real-time PCR. *Error bars* indicate standard deviations. All experiments were quadruplicated

and Bm$^{\text{WT-GP}}$ or Bm$^{\text{Bm65Rep-GP}}$ after 24 h p.t.. No significant difference was found between Bm$^{\text{WT-GP}}$- and Bm$^{\text{Bm65Rep-GP}}$-transfected cells through the infection period (Fig. 5).

Discussion

To date, most of identified baculovirus genes are involved in either the formation of capsid structure, ODV envelope, larval infectivity, DNA replication, or the transcription of late genes. According to their functions, the baculovirus genes can be classified into essential genes or auxiliary genes [12]. Essential genes are required for viral propagation, and auxiliary genes may regulate the transcription of viral genes. Some essential genes, such as *ac92* (*p33*), *ac76,* are critical for viral propagation, but do not affect the replication of viral DNA [13, 14], but genes such as DNA *polymerase* gene (*dnapol*), *ie1* [11, 15, 16] are essential for both virus propagation and DNA replication. In addition, *ie-2*, *lef-1*, *lef-2*, *lef-3*, *pe38*, and *p35* genes are identified to be involved in DNA replication [17].

The prediction of *Bm65* sequence suggests that *Bm65* is an early gene, implying that it may play roles in viral replication. Blast searching indicates that Bm65 is a member of GIY-YIG-like endonuclease superfamily, and its homologs are found in most lepidopteran-specific nucleopolyhedrovirus NPV, but not in lepidopteran-specific granuloviruses, hymenopteran-specific NPV, or dipteran-specific NPV. This suggests that *Bm65* gene may be host specific. In this study, *Bm65* transcript was detected as early as 6 h p. i. and remained detectable until 72 h p. i. by RT-PCR analysis (Fig. 2a). These results indicate that *Bm65* is an early gene, possibly involved in the transcriptional regulation of late genes.

Moreover, a *Bm65*-knockout bacmid was generated to investigate the role of *Bm65* in viral replication. The virus with a deletion in *Bm65* gene was unable to spread in BmN cells, which indicates that *Bm65* is essential for the propagation of BmNPV. The qPCR results showed that the Bm$^{\text{Bm65KO-GP}}$ has a similar DNA level with Bm$^{\text{WT-GP}}$ and Bm$^{\text{Bm65Rep-GP}}$ by 18 h p.t., and that DNA synthesis is greatly decreased in Bm$^{\text{Bm65KO-GP}}$ compared with Bm$^{\text{WT-GP}}$ at 24 h p.t.. It was reported that virus DNA replication in BV-infected cells starts at approximately 10–12 h p. i. [18]. Budded viruses are normally released from the infected cells by 24 h p.i, and then the next round infection starts. We showed that Bm65-knockout virus was unable to generate infectious BV (Fig. 4). Hence, we speculate that the different DNA replication levels between Bm$^{\text{Bm65KO-GP}}$ and Bm$^{\text{WT-GP}}$ after 24 h p.t were resulted from the consequences of secondary infection. It also indicated that *Bm65* was not essential for viral DNA replication.

In summary, our findings reveal that *Bm65* is essential for the propagation of *Bm*NPV, and that Bm65 deficiency does not affect the synthesis of viral DNA. Further research is required to investigate the action mechanism of Bm65 in viral propagation.

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