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Characterization of Ac*M*NPV with a deletion of *ac68* gene

Guohui Li · Jinwen Wang · Riqiang Deng · Xunzhang Wang

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Abstract *Orf68* (*ac68*) of *Autographa californica* multiple nucleopolyhedrovirus (Ac*M*NPV) is a highly conserved gene that codes a predicted 192-amino acid protein, but its function remains unknown. Results of the current study showed that *ac68* was transcribed from 3 to 96 h and the protein was detected from 36 to 96 h post infection. An *ac68* knockout bacmid was generated to investigate the role of the gene in baculovirus life cycle. Analyses of the production of infectious budded virus, occlusion bodies, and the formation of nucleocapsids revealed that there was no difference between the infection patterns of the mutant and its parent virus, or the *ac68* repair virus. Bioassay with *Trichoplusia ni* demonstrated that deletion of *ac68* did not affect Ac*M*NPV infectivity, but extended LT_{50} to a longer value. Taken together, our results indicated that the deletion did not affect viral propagation both in vitro and in vivo, but deletion of the gene may affect the virulence in *T. ni* larvae.

Keywords Baculovirus · orf68 · Ac*M*NPV

Introduction

Autographa californica multiple nucleopolyhedrovirus (Ac*M*NPV) is a member of the Baculoviridae, which are large, enveloped, double-stranded DNA viruses with circular covalently closed genomes ranging in size from 80 to 180 kbp [1]. Baculovirus life cycle typically involves the production of two virus forms, budded virus (BV) and occlusion-derived virus (ODV). Mature BVs exit nucleus and bud through the plasma membrane of infected cells, which are highly infectious for tissues of the hemocoel and for cultured cells. ODVs are occluded in polyhedra or occlusion bodies and are required for the oral infection. Ac*M*NPV is the most intensively studied member of baculovirus, with a genome of 134 kbp potentially encoding 154 genes [2]. To date, the functions of about 50% genes in Ac*M*NPV genome have been determined and according to their functions, the genes could be divided into genes essential for viral DNA replication, such as lef-1, lef-2, lef-3, p143, dnapol, and auxiliary genes that can regulate the transcription of viral genes in vivo.

Ac68 is a member of the 29 baculovirus core set genes [3], locating at 58,720–59,298 nt in Ac*M*NPV genome and encoding a putative protein of 192 amino acids with a predicted molecular mass of 22.3 kD. The amino acid identity between Ac68 protein and the homolog in *Rachiplusia ou* NPV is 95%; between Ac68 protein and the homolog in *Bombyx mori* NPV is 65%; in *Epiphyas postvittana* NPV is 44%; in *Hyphantria cunea* NPV is 44%; in *Choristoneura fumiferana* NPV is 43%; in *Choristoneura fumiferana defective* NPV is 42%. Sequence-based queries performed with interProScan program showed that *ac68* was a protein of unknown function.

The putative promoter region of *ac68*, as that of other early genes, contains a consensus early promoter motif ACATT, −15 nt upstream of ATG which might initiate the transcription during the early phase of viral replication. In addition, *ac68* gene also contains conserved sequences of TCATT and GATA, which are typical of most early genes, though locating at different sites, at +5 nt and +13 nt downstream of ATG, respectively [4]. Lulin Li et al. [5] reported that in a transient-expression assay system, *ac68* did not appear to affect late gene expression.

G. Li · J. Wang · R. Deng · X. Wang (✉)
State Key Laboratory of Biocontrol, Sun Yat-sen University, Guangzhou 510275, China
e-mail: wxz@sysu.edu.cn

In this study, an *ac68* gene knockout bacmid and a repair bacmid were generated to examine the role of this gene. The mutant was constructed from Ac*M*NPV bacmid by homologous recombination in *Escherichia coli* (*E. coli*). Green fluorescent protein (GFP) and polyhedrin were introduced into the mutant as markers. We found that the *ac68* deletion mutant had no striking phenotype in Sf-9 cells, including BV production, occlusion body formation, and viral morphogenesis.

Materials and methods

Virus, cells, and insects

Spodoptera frugiperda (Sf) 9 cells were grown at 27°C in Grace's medium supplemented with 10% fetal calf serum, penicillin, (50 U/ml) and streptomycin (50 μg/ml). Ac*M*NPV strain accompanying the Bac-to-Bac® system from Invitrogen was used in this study. The wild-type Ac*M*NPV bacmid ($vAc^{wt\text{-}GP}$) containing GFP and polyhedrin genes was constructed via introducing polyhedrin and GFP genes into the polyhedrin locus of Ac*M*NPV genome through transposition in *E. coli*. Larvae of *T. ni* were reared on an artificial diet at 28°C. Ac*M*NPV BV stocks were prepared by infecting third instar *T. ni* larvae with polyhedra and extracting hemolymph from infected insects 3 days p.i. as previously described [6].

Expression of Ac68 in *E. coli* and preparation of Ac68-specfic antibody

Two primers 68P1: 5′-ATGGATCCATGGTATTCATTATCGAT-3′ (*Bam*HI site was underlined) and 68P2: 5′-GCAAGCTTTTTAATTTTTGCTGCAA-3′ (*Hind*III site was underlined) were designed to amplify the ORF of *ac68* from Ac*M*NPV genomic DNA. The PCR product was cloned first into pMD18-T (TaKaRa) and then into the expression vector pET28a (Promega), which generated plasmid pET28a-*ac68* with 6× His-tag sequence at the N terminus. *E. coli* DE3 cells containing pET28a-*ac68* were grown to an optical density at about 0.6 of OD_{600} and induced by addition of 0.8 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG). After incubation for 12 h at 37°C, cells were harvested by centrifugation at 7,000*g* for 15 min at 4°C. The fusion protein present in the pellet was separated in 15% SDS-polyacrylamide gels and stained with Coomassie brilliant blue. The induced *ac68* band was excised directly and the antiserum was raised in rats according to the method of Sambrook et al. using Freund's adjuvant [7].

Total RNA isolation, reverse transcriptase (RT)-PCR

Total RNA was isolated from 1.6×10^6 mock-infected and Ac*M*NPV-infected Sf-9 cells (multiplicity of infection (m.o.i) of 5 plaque-formins unit (PFU) per cell) at 0, 0.5, 1, 3, 6, 12, 24, 48, 72, and 96 h p.i., respectively, using the TRIZOL® Reagent from Invitrogen.

After the RNA samples were treated with RNase-Free DNase, RT-PCR was performed using RNA-PCR-M-MLV Kit (Promega) with 3 μg RNA as the template per time point. First-strand cDNA synthesis was performed using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and the oligo(dT) according to the manufacturer's instructions. The cDNA product was amplified by the gene-specific primers 68P1 and 68P2. Ac*M*NPV *ie-1* gene and *vp39* gene were used as the control for early gene and late gene, respectively. The primer pair: ie-1U: 5′-ATGACGCAAATTAATTTTAACGC-3′ and ie-1D: 5′-ACAATTTAGTTTTTGTTCCG-3′ were designed for *ie-1;* the primer pair: vp39U: 5′-GCGCTAGTGCCCGTGGGTAT-3′ and vp39D: 5′-TTAGACGGCTATTCCTCCACCTGC-3′ were designed for *vp39*. The obtained PCR products were analyzed in 1.0% agarose gel. Control experiments in which no reverse transcriptase was added prior to the PCR step were performed to detect any possible viral DNA contamination. *ac68* RT-PCR products were gel purified and cloned into pMD18-T (TaKaRa) and sequenced with M13F(−47) or M13R(−48) primers.

In vitro expression of Ac68

For expression phase analysis of Ac68 protein, monolayer of Sf-9 cells were infected with mock or Ac*M*NPV (m.o.i. of 5). Cells were harvested at 0, 6, 12, 24, 36, 48, 72, and 96 h p.i., respectively. To confirm that no Ac68 protein was expressed in *ac68*-KO virus infected cells, monolayer of Sf-9 cells were infected with *ac68*-wt or *ac68*-KO virus (m.o.i. of 5). Cells were harvested at 48 and 72 h p.i., respectively. Then the cells were pelleted and resuspended in phosphate-buffered saline (PBS, pH 7.4), lysed in SDS-polyacrylamide loading buffer and analyzed by Western blot. *Ac68* antibodies and pre-serum were used at a dilution of 1:1,000. Immunoreactive proteins were visualized using goat anti-rat IgG and Horseradish Peroxidase.

Deletion of Ac*M*NPV ac68

An ac68 knockout Ac*M*NPV bacmid was generated by ET recombination system according to the manufacturer's instructions. We first generated a transfer vector in which the *ac68* locus region was replaced with Zeocin for antibiotic selection in *E. coli.* A 553-bp 5′ flank of *ac68* was amplified by PCR from the Ac*M*NPV bacmid using the primers 68US1: 5′-CGAAGCTTTCTTCCATGTCTTTGAAAGATTGC-3′ (*Hind*III site was underlined) and 68US2: 5′-CGGAATTCATTAACATTGACCGTTTGATCGT-3′ (*Eco*RI site was underlined). The PCR product was

digested with *Hind*III and *Eco*RI and then ligated into vector pBluescriptII-Zeocin (Li et al., unpublished) to generate the recombinant plasmid named pBluescriptII-US-Zeocin. With primers 68DS1: 5′-TGGGGATCCATGT TGCAGCAAAAATTAAAT-3′ (*Bam*HI site was underlined) and 68DS2: 5′-AAGAGCTCGGCAAATTAAAAT TAGCTG CGTC-3′ (*Sac*I site was underlined), a 528-bp 3′ flank of *ac68* was amplified by PCR from the Ac*M*NPV bacmid. The PCR product was digested with *Bam*HI and *Sac*I and cloned into plasmid pBluescriptII-US-Zeocin that was digested with *Bam*HI/*Sac*I to generate a final *ac68* knockout transfer vector named pBluescriptII-US-Zeocin-DS. This transfer vector was digested with *Hind*III and *Sac*I, and the resulting linear 1.8 kbp fragment containing Zeocin gene cassette and *ac68* flanking region was gel purified, resuspended in distilled water to a final concentration of 200 ng/μl.

An *ac68* knockout Ac*M*NPV bacmid was generated by ET recombination system according to the manufacturer's instructions. DH10B cells which contain Ac*M*NPV bacmid bMON14272 were transformed with plasmid pBAD-gbaA. Plasmid pBAD-gbaA can provide λ Red recombination function when it is induced by L-arabinose. Then DH10B electro-competent cells harboring plasmid pBAD-gbaA were made and electroporated with the 1.8 kbp fragments. The electroporated cells were incubated at 37°C for 1 h in 1 ml SOC medium, then 100 μl was spread onto low salt agar medium containing 25 μg/ml Zeocin, 100 μg/ml Amp, and 50 μg/ml Kanamycin. Plates were incubated at 37°C overnight, and colonies resistant to Zeocin and Kanamycin were selected, and target colony with *ac68* knockout bacmid was confirmed by PCR.

Deletion of *ac68* from its locus in Ac*M*NPV and correct insertion of Zeocin at the *ac68* locus were confirmed by PCR. The relative positions of the primer pairs are shown in Fig. 3b. Primer pair 68US1 and 68DS2 will amplify a 1,200 bp wild type fragment and an 1,873 bp knockout fragment. Primer pair Zeo-U and Zeo-D were used to detect the correct insertion of the Zeocin gene cassette. Primer pair 68P1 and 68P2 was used to confirm the deletion of the *ac68* gene. Primer pairs 68US1/Zeo-D, Zeo-U/68DS2, and 68P1/Zeo-D were used to examine the junction between the upstream or downstream flanking region and Zeocin.

Construction of knockout, repair, and wt Ac*M*NPV bacmids containing polyhedrin and GFP

To construct repair bacmid, a 892-bp fragment containing *ac68* gene with native promoter and polyadenylation signal was amplified by PCR using primers 68REP-U: 5′-CGTCTAGAAACAACTTTGTACAAACC-3′ (*Xba*I site was underlined) and 68REP-D: 5′-CGCTCGAGTTATTTAAT TTTTGCT-3′ (*Xho*I site was underlined). The PCR product was digested with *Xba*I/*Xho*I and ligated with pFB-ieGP [8], which was also digested with the same enzymes, to generate pFB-ieGP-*ac68*. To prepare bacmid for transposition, the helper plasmid pMON7124 conferring resistance to tetracycline and encoding a transposase was transformed into DH10B harboring the *ac68* knockout bacmid. Then these cells were transformed with donor plasmid pFB-ieGP or pFB-ieGP-*ac68* to generate *ac68* bacmid $vAc^{ac68KO\text{-}GP}$ or *ac68* repair bacmid $vAc^{ac68REP\text{-}GP}$. In addition, electro-competent DH10B cells containing pMON7124 helper plasmid and bacmid bMON14272 were transformed with pFB-ieGP to generate a control virus named $vAc^{wt\text{-}GP}$. The transformed cells were incubated at 37°C for 4 h in 1 ml SOC medium with moderate shaking, spread onto LB agar medium containing 25 μg/ml Zeocin, 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 resistant to Zeocin, Kanamycin, Gentamicin, and Tetracycline were selected and confirmed by PCR analysis.

Transfections and fluorescence microscopy

Sf-9 cells (10^6 cells/well) were seeded into six-well culture plates and were incubated at 27°C for 16–24 h before transfection. DNA of three bacmid constructs was isolated from each resulting *E. coli* which had discarded the helper plasmid pMON7124 by resistance crossing. Equimolar amounts of bacmid DNA were transfected into Sf-9 cells using Cellfectin Reagent (Invitrogen Life Technology). Bacmid DNA was mixed with 6 μl cellfectin in 200 μl non-serum Grace's medium and incubated at 27°C for 45 min, then the mixed DNA-cellfectin solution was added to 800 μl non-serum Grace's medium and overlaid onto the seeded Sf-9 cells. After incubation for 4 h, the supernatant was removed and the cells were washed twice with non-serum Grace's medium. Finally the cells were incubated at 27°C with 2 ml Grace's medium containing 10% fetal bovine serum. GFP expression and polyhedra formation were examined through fluorescence microscopy to study the role of $vAc^{ac68KO\text{-}GP}$, $vAc^{ac68REP\text{-}GP}$, and $vAc^{wt\text{-}GP}$ on Sf-9 cells.

Analysis of viral growth curve

To assess whether *ac68* is required for virus production and determine the replication kinetics of the virus constructed, a virus growth curve analysis was performed as described previously [9]. For this experiment, Sf-9 cells were transfected in triplicate with each bacmid DNA (~2 μg/well), and the culture supernatants were collected from various time points. BV titers were determined using a $TCID_{50}$ end-point dilution assay described previously [8]. Virus

infection was determined by monitoring GFP expression with fluorescence microscopy. A well of the culture plate was identified as positive if there were one or more cells expressing GFP.

Electron microscopy

For electron microscopy, Sf-9 cells (5×10^6 per 100-mm-diameter tissue culture dish) were infected with BV ($vAc^{ac68KO\text{-}GP}$ or $vAc^{wt\text{-}GP}$) at a MOI of 5. At 24, 48, and 72 h p.i., cells were harvested, washed once with Grace's medium lacking fetal serum, and centrifuged at 3,000*g* for 10 min. Then cells were fixed, dehydrated, embedded, sectioned, and stained as described previously [8]. Samples were observed under a JEM-100CXII transmission electron- microscope at an accelerating voltage of 80 KV.

Bioassays

In order to determine whether *ac68* deletion has any effect on the infectivity, bioassays were performed as described previously [10]. BVs diluted in Grace's medium at the concentration of 2×10^6 $TCID_{50}$/ml were injected into the hemocoel of fourth-instar *T. ni* larvae. PIBs were harvested from dead larvae, purified by centrifugation, and resuspended in double distilled water as described previously [6]. To determine the LD_{50}, 10 µl samples containing 0 (control), 2×10^2, 1×10^3, 2×10^3, 1×10^4, and 2×10^4 PIBs, respectively, were applied onto a small piece of artificial diet. Newly molted third-instar *T. ni* larvae were reared at 27°C individually in 12-well plates and were infected by allowing them to feed on a diet contaminated with above concentrations of *ac68* wt or *ac68* deleted PIBs. Fresh diet was added into the wells when all the virus-contaminated diet was consumed. Control experiment was made with uncontaminated diet and each experiment was made three times repeatedly. Forty five larvae per dosage were used in the experiment. Mortality was recorded at 12 h intervals until larvae died or pupated. The data were analyzed by probit analysis. The LT_{50} were determined in *T. ni* larvae by droplet feeding assays as previously described [11]. Newly molted third-instar *T. ni* larvae were starved for 6 h, then were fed a solution containing 1% sucrose (w/v), 0.01% blue food coloring (FD and C Blue NO.1), as well as PIBs (1×10^8 PIBs/ml). Orally infected larvae were maintained individually in the dark at 27°C and mortality was recorded every 8 h until larvae died or pupated. Thirty six larvae were used per treatment group and the experiments were made three times repeatedly. The data were analyzed by Kaplan-Meier estimator in the bioassays.

Results

RT-PCR analysis of ac68 transcripts

To determine the temporal expression of *ac68* transcripts at early, late, and very late phases of virus infection, RT-PCR was performed using total RNA isolated from Ac*M*NPV-infected Sf-9 cells at different time points as template. We took advantage of Ac*M*NPV *ie-1* gene and *vp39* gene as the controls for the early gene and late gene, respectively. As expected, a 1722 bp *ie-1* transcript was amplified by *ie-1* specific primers from 0.5 h p.i. to 72 h p.i. and a 1,041 bp *vp39* transcript was detectable from 12 h p.i. to 96 h p.i. when amplifying by *vp39* specific primers (Fig. 1). Using the gene-specific primers 68P1 and 68P2, a single band with an expected size of 592 bp was amplified at 3 h p.i., which remained detectable up to 96 h p.i. (Fig. 1), indicating that *ac68* is an early gene according to the current view on early versus late transcription [12], in agreement with the sequence analysis of *ac68*, in which an early baculovirus transcription initiation motif ACATT was found at −15 nt upstream of ATG. The RT-PCR products obtained were cloned into pMD18-T and sequenced. The obtained sequence matched, as expected, the *ac68* sequence. No RT-PCR product was detected in mock-infected Sf-9 cells.

Immunodetection of Ac68 in Ac*M*NPV-infected cells

Antibody was prepared by immunization of rats with purified Ac68 produced in *E. coli*. Western blot analysis of

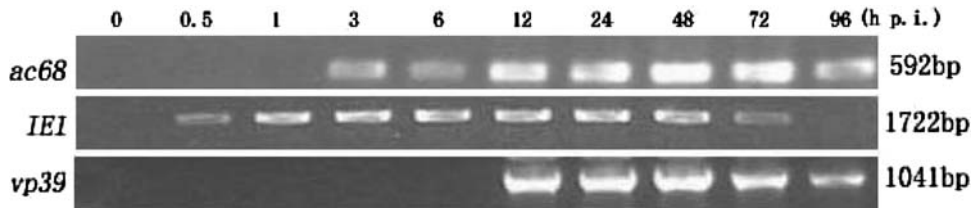

Fig. 1 RT-PCR analysis of *ac68* transcription. Total RNA was extracted from Ac*M*NPV-infected Sf-9 cells at different times post infection (p.i.). PCR products of different genes are indicated on the left. Times p.i. are indicated above the lanes. The sizes (in bp) of different PCR products are indicated on the right. IE1 and vp31 are positive controls for early and late genes

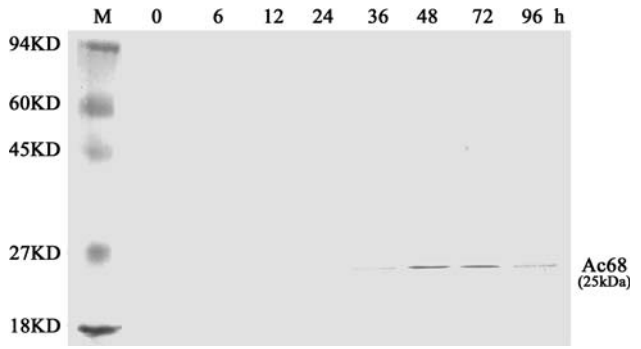

Fig. 2 Time course of Ac68 expression in Ac*M*NPV-infected Sf-9 cells. Times p.i. are indicated above the lanes. The pre-stained protein standards are indicated on the left and the corresponding band to Ac68 is indicated on the right

extracts from Ac*M*NPV-infected cells revealed a specific 25 kDa band when using the Ac68 antiserum (Fig. 2), a little larger than the predicted size of Ac68, which was 22.3 kDa, implying possible posttranslational modification of the protein.

Construction of ac68 knockout Ac*M*NPV bacmid

To investigate the function of *ac68* during the viral infection cycle, we generated a bacmid by ET recombination system according to the manufacturer's instructions, in which a 120-bp fragment (nt 59,179–59,298) of the *ac68* gene coding region was replaced by Zeocin gene (Fig. 3a). The region contains a strong hydrophobicity domain as predicted using the Bioedit 7.0 software.

The deletion of *ac68* was confirmed by PCR with several primer pairs (Fig. 3c). Primer pair 68US1 and 68DS2 produced an 1,873-bp product from the mutant genome, in comparison with a 1,200-bp product from the wild-type genome. Primer pair 68US1/Zeo-D produced no PCR product in vAc^{wt}, but a fragment of 1,345-bp in vAc^{ac68KO}. Primers Zeo-U/Zeo-D produced no PCR product in vAc^{wt}, but a fragment of 792-bp in vAc^{ac68KO}. Primers Zeo-U/68-DS2 produced no PCR product in vAc^{wt}, but a fragment of 1,300-bp in vAc^{ac68KO}. Primers 68P1/68P2 produced a 592-bp fragment in vAc^{wt}, but no PCR product in vAc^{ac68KO}. Primers 68P1/Zeo-D produced no PCR product, but a 1,259-bp fragment in vAc^{ac68KO}. These results demonstrated that Zeocin gene cassette had replaced *ac68* gene in Ac*M*NPV bacmid successfully and no intact *ac68* gene cassette existed in the vAc^{ac68KO} genome.

Immunodetection of Ac68 in the extracts from ac68-wt or ac68-KO infected cells

To confirm that the truncated Ac68 is not expressed in the *ac68*-KO virus infected cells, antibodies against the Ac68 protein was used to perform western blot analysis. The result showed that a 25 kDa band was detected in the extracts from *ac68*-wt infected cells, while no similar band in the extracts from *ac68*-KO infected cells was detected, indicating that the truncation has successfully blocked *ac68* gene expression (Fig. 4).

Construction of knockout, repair, and wt Ac*M*NPV bacmids containing polyhedrin and GFP (green fluorescent protein)

Transposition events were confirmed by PCR with primer set PUC/M13 just outside of the transposition locus, which produced PCR fragment with expected sizes from specific bacmid constructs (data not shown). In addition, GFP expression and occlusion body formation in bacmid DNA transfected Sf-9 cells as examined by fluorescence microscopy further confirmed the accomplishment of the transposition events.

Analysis of knockout, repair, and wt Ac*M*NPV replication in infected Sf-9 cells

To further assess whether *ac68* is required for virus production, the bacmids with the *ac68* gene removed ($vAc^{ac68KO\text{-}GP}$), repaired ($vAc^{ac68REP\text{-}GP}$), or the wild-type control ($vAc^{wt\text{-}GP}$) were transfected into Sf9 cells. Fluorescence could be detected at 24 h p.t., and at this time point all cultures exhibited isolated GFP expression. At 72 h p.t. or 96 h p.t., approximately 100% transfected cells were observed expressing GFP with no difference among the cells transfected with different bacmids (Fig. 5a). Supernatants from cultures transfected with $vAc^{wt\text{-}GP}$, $vAc^{ac68KO\text{-}GP}$, or $vAc^{ac68REP\text{-}GP}$ at 96 h p.t. were collected and the titers were determined by $TCID_{50}$ (50% Tissue Culture Infective Dosage). The collected supernatants were then used to infect Sf-9 cells at a MOI of 5. Fluorescence was observed at 48 h p.i. in all cultures infected with the supernatants (data not shown).

Occlusion bodies appeared in all cultures infected with $vAc^{wt\text{-}GP}$, $vAc^{ac68KO\text{-}GP}$, or $vAc^{ac68REP\text{-}GP}$ viruses at around 48 h p.i. And occlusion bodies were observed in almost every single cell infected with $vAc^{wt\text{-}GP}$, $vAc^{ac68KO\text{-}GP}$, or $vAc^{ac68REP\text{-}GP}$ viruses at 72 h p.i. (Fig. 5b). The above results indicated that *ac68* deletion did not affect infectious budded virus generation and polyhedra formation.

To further assess the effect of *ac68* deletion on virus replication and determine the replication kinetics of the virus constructs, the time-course of budded virus production after transfection with the three bacmids was studied. At 24, 48, 72, and 96 h p.t. time points, the supernatants were collected and the BV titers were determined by $TCID_{50}$ end-point dilution assay on Sf-9 cells. The result

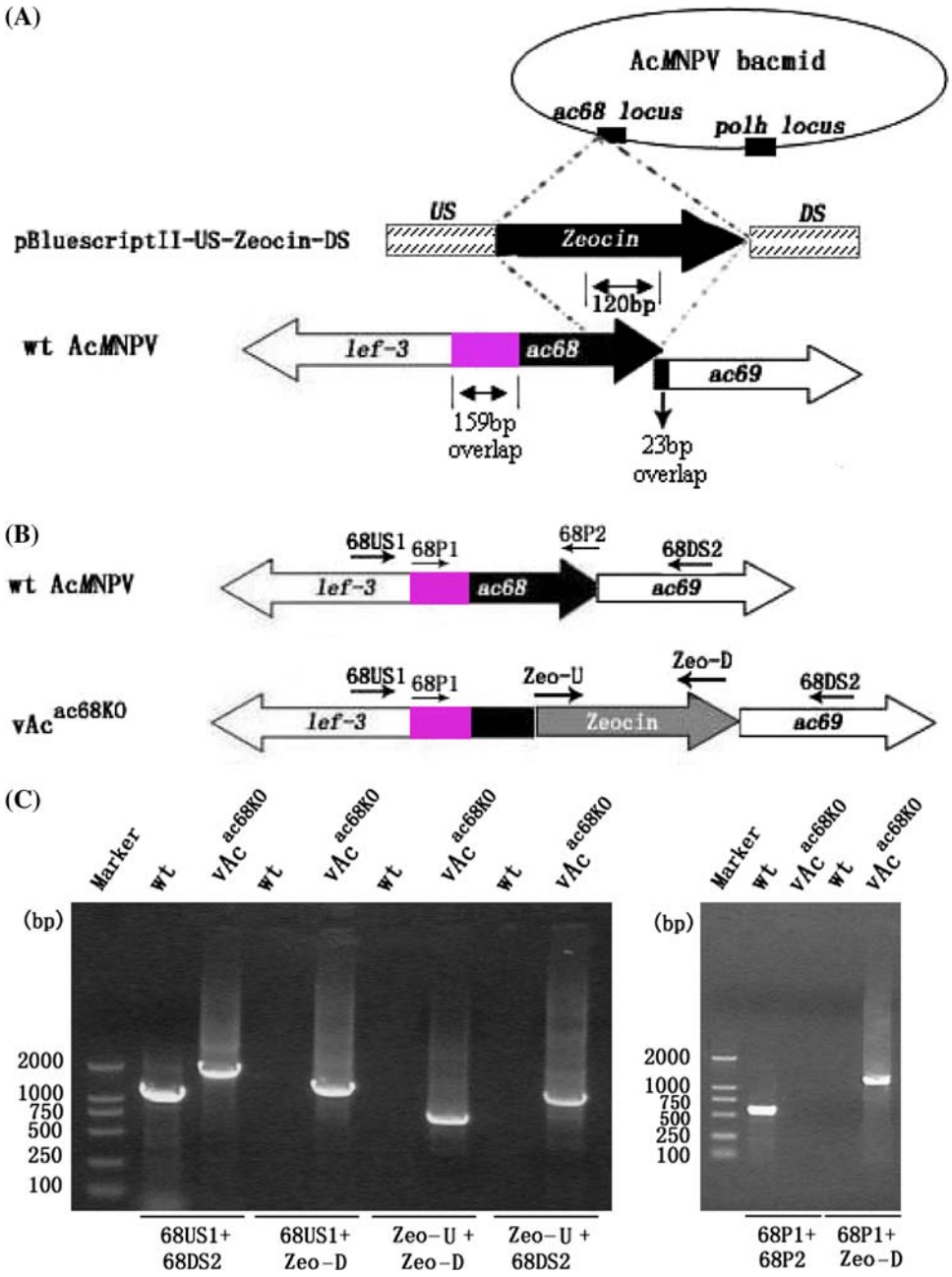

Fig. 3 Strategy for construction of *ac68* knockout Ac*M*NPV bacmid and confirmation by PCR analysis. (**a**) Schematic diagram showing the structure of *ac68* locus in wild-type and *ac68*-deleted virus and the replacement of a 120 bp fragment by Zeocin resistance gene. (**b**) Positions of primer pairs used in the confirmation of the *ac68* gene deletion and correct insertion of Zeocin resistance gene cassette. (**c**) PCR analysis of different primer pairs. Marker: DNA marker with sizes indicated. The virus templates are shown above each lane and the primer pairs used are shown below

revealed steady increase in virus production and almost the same slope of the growth curves for the three viruses (Fig. 5c).

Electron microscopic analysis of mock, wt, knockout virus-infected Sf-9 cells

To further analyze whether the deletion of *ac68* has any effect on virus morphogenesis, we examined the cells infected with BV ($vAc^{wt\text{-}GP}$ or $vAc^{ac68KO\text{-}GP}$) by electron microscopy. As expected, the sections of $vAc^{wt\text{-}GP}$ infected Sf-9 cells showed typical cytological changes and viral morphogenesis of NPV infection in the field of vision, and no morphological difference was found between cells infected with wild type and *ac68* knockout viruses. Typical virogenic stroma, the putative site of nucleocapsids assembly with an electron-dense lattice-like structure, could be found easily within the nucleus of the *ac68* knockout or wild type virus-infected cells (data not shown), with a lot of nucleocapsids at the edges of the electron-dense virogenic stroma, which did not exist in mock-infected cells (data not shown). At 48 h p.i., many enveloped virions containing multiple nucleocapsids could be observed in the sections, which formed in the ring zone and were embedded in the polyhedra. The number, as well as the sizes and shapes, of polyhedra in the *ac68* knockout virus-infected cells were

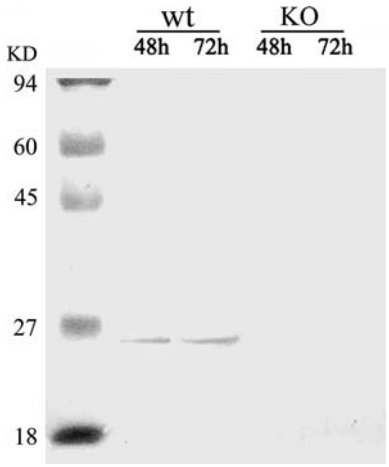

Fig. 4 Western blot analysis of Ac68 in Sf-9 cells infected with Ac-wt or *ac68*-KO virus. Virus used for infection and times p.i. are indicated above the lanes. The pre-stained protein standards are indicated on the left

indistinguishable to that in wild type virus-infected cells (data not shown). These observations suggested that the deletion of *ac68* gene had no effect on the occlusion body morphogenesis.

Effect of ac68 deletion on Ac*M*NPV infectivity for Trichoplusia ni larvae

The infectivities of $vAc^{wt\text{-}GP}$ and $vAc^{ac68KO\text{-}GP}$ were determined for newly molted fourth *T. ni* larvae in 50% lethal dose (LD_{50}) and 50% lethal time (LT_{50}) bioassays. The results of dose-mortality (LD_{50}) assessing showed that there was no significant difference between $vAc^{wt\text{-}GP}$ and $vAc^{ac68KO\text{-}GP}$ viruses (Table 1), but it took 16 h longer for *ac68* deleted virus to kill the *T. ni* larvae than did for *ac68* wt or *ac68* repair virus in LT_{50} bioassay (Table 2). There is significant difference ($P < 0.01$) between the LT_{50} of $vAc^{ac68KO\text{-}GP}$ and that of $vAc^{wt\text{-}GP}$ or $vAc^{ac68REP\text{-}GP}$ by the analysis of Tukey HSD.

Discussion

Ac68 is a highly conserved gene in lepidopteron NPVs, but sequence-based queries performed with InterProScan program did not show significant sequence similarities to non-baculovirus proteins with known functions.

Ac68 locates downstream of *ac67* (lef-3) gene with 159 overlap nucleotides at 5′ terminus; lef-3 is a single stranded DNA-binding protein which is necessary for viral DNA replication [13]. Primer extension analysis and S1 nuclease protection assays revealed that *lef-3* transcription initiated about 280 bp upstream of the first ATG condon [14], i.e. 439 nucleotides in the *lef-3* transcription overlap with the ac68 gene. In order to keep the *lef-3* gene intact and its function, we disrupted only the putative 3′ terminal portion of ac68 gene, which represents a hydrophobic domain as predicted with the Bioedit 7.0 soft. A 792-bp DNA fragment containing the complete Zeocin gene was inserted into the deleted locus, which interrupted the conformation of Ac68 protein, making it extremely unlikely that the fusion protein could maintain the activity of Ac68 protein.

In this report, the mutant virus was examined for phenotype. Successful propagation, infectious progeny virus generation, formation of occlusion bodies, and polyhedra morphogenesis, and the virus growth curve of the *ac68* knockout virus indicated little or no effect of *ac68* deletion on virus replication in tissue culture and in *T. ni* larvae.

The *ac68* gene had originally been identified in transient-expression assays as unnecessary for late gene expression [5], which was confirmed by our results. In this study, *ac68* mRNA was detected from 3 to 96 h p.i., and was most abundant from 24 to 72 h p.i., identifying *ac68* as an early gene that is expressed in both early and late phases, though late conserved motif in its 5′ flanking region could not be found. To date, most early genes that have been discovered in Ac*M*NPV genome are essential to the production of BV such as *ac38*, EXON0, lef-3 [15–17]. Some early genes such as pk2, lef-6, and *ac18* have little effect on the production of BV, which are regarded as auxiliary genes [18–20]. Auxiliary genes are not essential for viral replication but play a potential role in modulating or regulating late transcription in the life cycle of Ac*M*NPV. It is accepted generally that auxiliary genes of baculovirus can function at the cellular level, i.e. protein kinase 2 (*pk2*), or at the organismal level, i.e. cathepsin (*cath*) [20]. Lef-6 is an early gene which is not essential for either viral DNA replication or late gene transcription, but the absence of lef-6 resulted in a substantial delay in the onset of late transcription [19]. *Ac18* is also an early gene and is not essential for the propagation of Ac*M*NPV, but it affects the efficient infectivity in *T. ni* larvae [20]. In this study, *ac68* was identified as an early gene that was not required for viral replication in cell culture or in *T. ni* larvae, though *ac68* accelerated the mortality of infected insect hosts by approximately 16 h, which might provide a selective advantage for the virus.

Western blot analysis confirmed that no Ac68 protein was expressed in the ac68-KO virus infected cells. In ac68-wt virus infected cells, a 25 kDa band was detected from 36 to 96 h after infection.

The deduced amino acid sequence analysis showed six phosphorylation sites in the Ac68 protein with NetPhos software (http://www.expasy.ch), speculating Ac68 protein relevant to phosphorylation, which plays a regulatory role

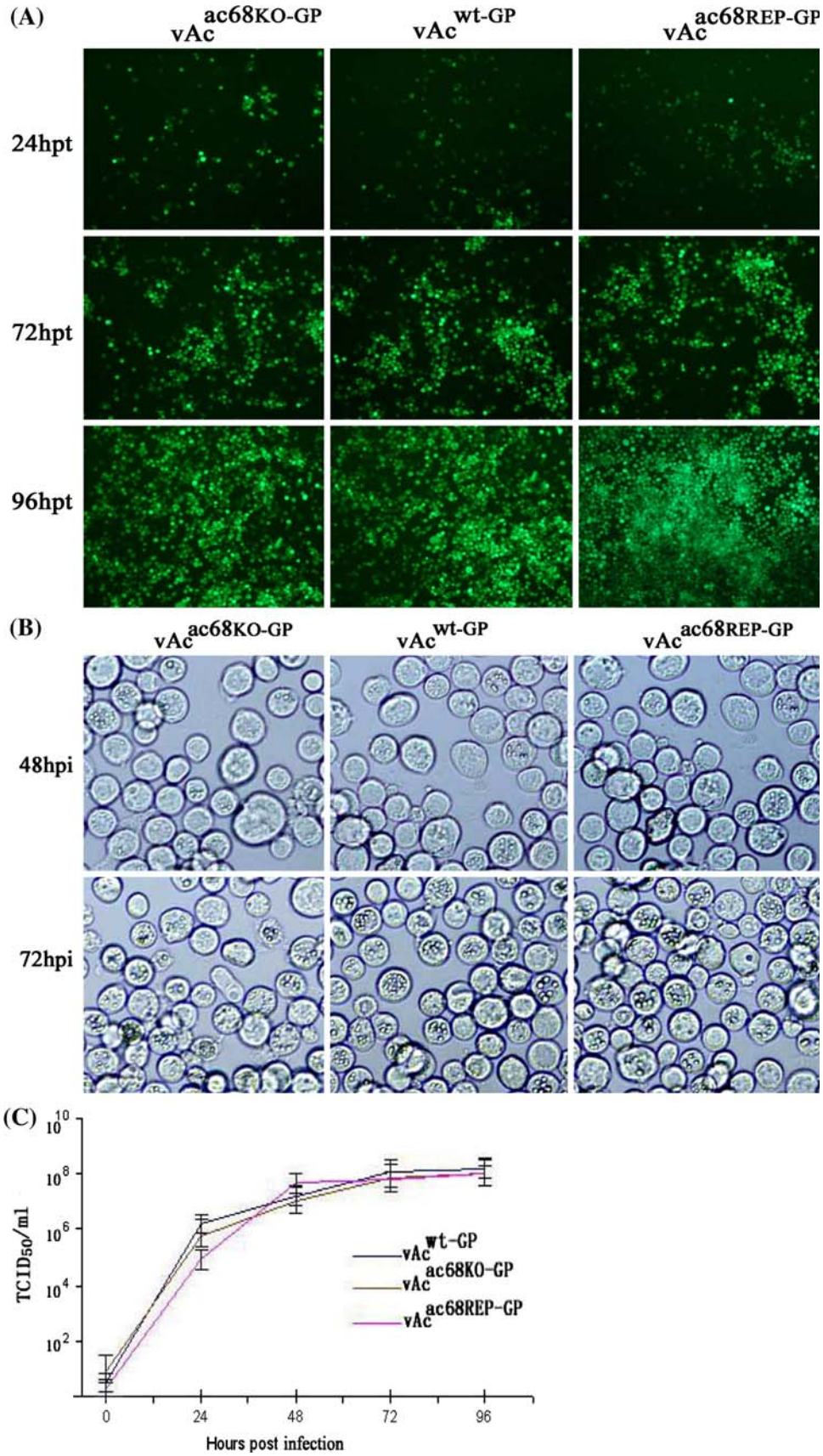

Fig. 5 (**a**) Fluorescence micrographs of Sf-9 cells transfected with bacmids $vAc^{ac68KO\text{-}GP}$, $vAc^{wt\text{-}GP}$, or *ac68* repair $vAc^{ac68REP\text{-}GP}$. Bacmids used for transfection were indicated above and the times after transfection were indicated on the left. (**b**) Micrographs of Sf-9 cells infected with $vAc^{ac68KO\text{-}GP}$, $vAc^{wt\text{-}GP}$, or $vAc^{ac68REP\text{-}GP}$ virus, showing polyhedra in the infected cells. Viruses used for infection were indicated above and the time post infection was indicated on the left. (**c**) Viral growth curves. Sf-9 cells were transfected with bacmids $vAc^{ac68KO\text{-}GP}$, $vAc^{wt\text{-}GP}$, or $vAc^{ac68REP\text{-}GP}$, and the supernatants were collected at the indicated time points and the titers were determined by $TCID_{50}$ assay. Each sample was performed in triplicate

Table 1 Dose-mortality of $vAc^{wt\text{-}GP}$ and $vAc^{ac68KO\text{-}GP}$ for third-instar *T. ni* larvae

Virus	LD_{50} (PIBs per larvae)	95% Fiducial limit (PIBs per larvae)	
		Lower	Upper
$vAc^{wt\text{-}GP}$	2.41×10^3	1.33×10^3	3.58×10^3
$vAc^{ac68KO\text{-}GP}$	2.28×10^3	1.24×10^3	3.19×10^3

Table 2 Time-mortality of $vAc^{ac68KO\text{-}GP}$, $vAc^{wt\text{-}GP}$ and $vAc^{ac68REP\text{-}GP}$ for third-instar *T. ni* larvae

Virus	LD_{50} (h)	95% Fiducial limit (h)	
		Lower	Upper
$vAc^{ac68KO\text{-}GP}$	88	86.41	89.59
$vAc^{wt\text{-}GP}$	72	68.39	75.62
$vAc^{ac68REP\text{-}GP}$	72	67.19	76.82

in the life cycle of Ac*M*NPV. Further experiments will help to clarify the posttranslational modification and the specific function of Ac68 protein in the life cycle of Ac*M*NPV.

In addition, *ac68* gene is adjacent to orf69 with 23 overlap nucleotides at C terminus. The Ac*M*NPV orf69 gene is a late gene and encodes a Methyltransferase (MTase1). Early transcripts of *ac68* gene contains at least the N-terminal part of the orf69 open reading frame, which could potentially direct the synthesis of MTase1 during the early phase of infection [21]. Although the disruption site was in the C-terminal coding region of *ac68*, it was still within 5′-TIR of *ac69*. Therefore the disruption affects the expression of both *ac68* and *ac69*. Fortunately Wu and Guarino [21] had reported that the disruption of *ac69* had no effect on the production of BV.

Our results, together with transient-expression assays [5] and the reports by Wu and Guarino [21], demonstrated that Ac68 protein may be an accessory factor or a minor component in regulating or modulating the transcription of late gene during the infection cycle. Therefore, it will be of great interest to more precisely elucidate the molecular mechanism of action of *ac68* gene in the life cycle of Ac*M*NPV.

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