Helicoverpa armigera nucleopolyhedrovirus occlusion-derived virus-associated protein, HA100, affects oral infectivity in vivo but not virus replication in vitro

Sijiani Luo,1 Yanfang Zhang,1 Xushi Xu,1,2 Marcel Westenberg,3 Just M. Vlak,3 Hualin Wang,1 Zhihong Hu1 and Fei Deng1

1State Key Laboratory of Virology and CAS Key Laboratory of Agricultural and Environmental Microbiology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, PR China
2Faculty of Biosciences, Nanjing Normal University, Nanjing 210097, PR China
3Laboratory of Virology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

ORF100 (ha100) of Helicoverpa armigera nucleopolyhedrovirus (HearNPV) has been reported as one of the unique genes of group II alphabaculoviruses encoding a protein located in the occlusion-derived virus (ODV) envelope and nucleocapsid. The protein consists of 510 aa with a predicted mass of 58.1 kDa and is a homologue of poly(ADP–ribose) glycohydrolase in eukaryotes. Western blot analysis detected a 60 kDa band in HearNPV-infected HzAM1 cells starting at 18 h post-infection. Transient expression of GFP-fused HA100 in HzAM1 cells resulted in cytoplasmic localization of the protein, but after superinfection with HearNPV, GFP-fused HA100 was localized in the nucleus. To study the function of HA100 further, an ha100-null virus was constructed using bacmid technology. Viral one-step growth curve analyses showed that the ha100-null virus had similar budded virus production kinetics to that of the parental virus. Electron microscopy revealed that deletion of HA100 did not alter the morphology of ODVs or occlusion bodies (OBs). However, bioassays in larvae showed that the 50 % lethal concentration (LC50) value of HA100-null OBs was significantly higher than that of parental OBs; the median lethal time (LT50) of ha100-null OBs was about 24 h later than control virus. These results indicate that HA100 is not essential for virus replication in vitro. However, it significantly affects the oral infectivity of OBs in host insects, suggesting that the association HA100 with the ODV contributes to the infectivity of OBs in vivo.

INTRODUCTION

Baculoviruses are large, dsDNA viruses pathogenic to a variety of insect species and many have been developed as alternative insecticides in agriculture and forestry. Helicoverpa armigera nucleopolyhedrovirus (HearNPV; also called HaSNPV) was isolated from diseased larvae in the province of Hubei, China, and has been developed as a pesticide against the cotton bollworm in China (Sun et al., 1998). As an initial step to genetically engineer HearNPV to improve its insecticidal activity, the genome of an in vivo-cloned strain, HaSNPV-G4 (Chen et al., 2001), was sequenced. Many HearNPV genes have been investigated and their functions annotated (Dong et al., 2005; Fang et al., 2006; Pan et al., 2007; Peng et al., 2010; Song et al., 2008; Tan et al., 2008; Wu et al., 2005). As such, HearNPV has become one of the best-characterized members of group II of the genus Alphabaculoviruses.

Recently, HA100, encoded by ORF100, was identified as an occlusion-derived virus (ODV)-associated protein using proteomics technology. Homologues of ha100 are conserved among group II alphabaculoviruses, suggesting an important role for HA100 in the biology of this group of baculoviruses. HA100 has homology with poly(ADP–ribose) glycohydrolase (PARG), a ubiquitously expressed exo- and endoglycohydrolase in eukaryotic cells (Deng et al., 2007). This enzyme, which is present in various isoforms in eukaryotes, is involved in the cleavage of ADP–ribose polymers. The enzyme may play a physiological role in modulating chromosome structure, transcription, DNA repair and apoptosis (Bonicalzi et al., 2005). As most studies on PARG have focused on mammals and insects, it is of interest to determine the function of HA100 in the baculovirus multiplication cycle and why it is associated with ODVs.
In this paper, the function of HA100 was studied with particular reference to its expression and subcellular localization. We also constructed an ha100-null recombinant virus to investigate its role in virus biology, infectivity and replication. These studies provide further insights into the function of this unique protein in group II alphabaculoviruses.

RESULTS

Expression analysis
The ha100 gene encodes a protein of approximately 58 kDa. A late transcription initiation motif, ATAAP, was found 37 nt upstream of the ATG translation start codon of ha100 (Chen et al., 2001) suggesting that ha100 is a late gene. HA100 was detected from 18 to 96 h post-infection (p.i.) as a protein band of about 60 kDa by Western blot analysis (Fig. 1), which indicated that ha100 is indeed a late gene and is expressed at a time when ODV proteins are made and assembled into ODVs.

Localization of HA100 protein in insect cells
To study the localization of HA100 in insect cells, pIZ/V5-egfp/ha100 was constructed, in which the egfp gene is fused in frame to the N terminus of ha100, and used to transfect HzAM1 cells. The intracellular localization of HA100 was examined by confocal laser-scanning microscopy. The GFP-fused HA100 protein by itself was localized exclusively in the cytoplasm (Fig. 2c). When HzAM1 cells were superinfected with HearNPV, most GFP-fused HA100 was found in the nucleus (Fig. 2d). As a negative control, EGFP downstream of the same promoter showed diffuse fluorescence in the cytoplasm and nucleus in uninfected (Fig. 2a) or superinfected (Fig. 2b) HzAM1 cells.

Construction and infection of recombinant HearNPV viruses
Three recombinant bacmids, an ha100 knockout bacmid (bHaBacha100-KO-ph) where the polyhedrin gene (ph) was reinserted into the ph locus, a repair bacmid (bHaBacha100-REP-ph) where ha100 under the control of its own promoter was inserted at the ph locus and a wild-type HearNPV bacmid containing egfp and ph (bHaBac-egfp-ph) as a positive control were constructed as described in Methods (Fig. 3). All recombinants, carrying egfp driven by the hsp70 promoter at the empty ha100 locus, were authenticated by PCR and restriction enzyme analyses. Supernatant fluid from cells transfected with the ha100 knockout bacmid, bHaBacha100-KO-ph, was infectious to healthy HzAM1 cells [Fig. 4a, panels (i) and (iv)], indicating that HA100 is not essential for virus replication. Transfection and infection of the repair bacmid, bHaBacha100-REP-ph [Fig. 4a, panels (ii) and (v)], and positive-control bacmid, bHaBac-egfp-ph [Fig. 4a, panels (iii) and (vi)], resulted in a similar infection process to that for bHaBacha100-KO-ph.

Western blot analysis showed that HA100 was detectable at 72 h.p.i. in cells infected with the virus vHaBacha100-REP-ph, vHaBac-egfp-ph or wild-type HearNPV, with a band of ~58 kDa (Fig. 4b). The knockout virus vHaBacha100-KO-ph did not synthesize the HA100 protein at a detectable level. Likewise HA100 was also not found in uninfected cells. Equal numbers of cells were used for blotting. These results confirmed that the recombinants were constructed correctly.

One-step growth curves of recombinant viruses
HzAM1 cells were infected with vHaBacha100-KO-ph, vHaBacha100-REP-ph and vHaBac-egfp-ph at the same m.o.i. One-step growth curves of budded viruses (BV) are shown in Fig. 4(c). The results showed that vHaBacha100-KO-ph, vHaBacha100-REP-ph and vHaBac-egfp-ph had similar kinetics of BV production and that the titres of vHaBacha100-KO-ph were similar to that of the parental virus at similar times after infection. Statistical analysis of each time point (12, 24, 48, 72 and 96 h.p.i.) for these three recombinants viruses revealed that there was no significant difference between HA100-null virus and HA100-repaired virus or the parental virus (P>0.05).

Electron microscopic observations
To analyse further whether the deletion of ha100 had any effect on virus morphogenesis, electron microscopic analysis was performed on cells infected with the different viruses and analysed at 72 h.p.i. (Fig. 5). Cells infected with vHaBacha100-KO-ph and vHaBacha100-REP-ph exhibited cytopathological changes and viral morphogenesis typical of infection by a single nucleopolyhedrovirus and the two

![Fig. 1. Expression analysis of HearNPV ha100.](http://vir.sgmjournals.org)
viruses were morphologically indistinguishable (Fig. 5a, b). The morphology of purified occlusion bodies (OBs) (vHaBac100-KO-ph; Fig. 5c) was similar to that of control-virus OBs (Fig. 5d). These studies appeared to demonstrate that HA100 does not play a major role in ODV morphogenesis and occlusion into OBs.

Fig. 2. Subcellular localization of EGFP-fused HA100. Confocal fluorescence analysis was employed to study the localization of the protein in infected cells. HzAM1 cells were transfected with pIZ/V5-egfp (a, b) or pIZ/V5-egfp/ha100 (c, d) followed by superinfection (b, d) with HearNPV. Fluorescent microscopy was performed at 72 h p.i. Left panels are images under fluorescent microscopy and right panels show the overlay results under fluorescent and visible microscopy. Bars, 10 μm.

Fig. 3. Construction of recombinant bacmids. The chloramphenicol acetyltransferase (cat) and egfp genes flanked by ha100 sequences (57 bp homologous arm) were used for homologous recombination to generate bHaBac100-KO. The ph gene was reintroduced into bHaBac100-KO to generate bHaBac100-KO-ph. The ha100 and ph genes under their native promoters were inserted into the ph locus of HaBacHZ8 by Tn7-mediated transposition to generate bHaBac100-REP-ph. bHaBac-egfp-ph was a control virus containing EGFP as the marker. Gm', Gentamicin resistance gene.
Biological activity of vHaBacha100-KO-ph

To determine the 50% lethal concentration (LC50) values of vHaBacha100-KO-ph and vHaBacha100-REP-ph, third-instar H. armigera larvae were infected orally with selected doses of OBs and monitored for mortality. The LC50 value of vHaBacha100-KO-ph was $1.2 \times 10^4$ ml$^{-1}$. In contrast, the LC50 of vHaBacha100-KO-ph was $6.8 \times 10^5$ ml$^{-1}$, which was significantly higher than HA100-repaired OBs. Statistical analysis also indicated that the LC50 value of vHaBacha100-KO-ph was significantly higher than that of vHaBacha100-REP-ph (ratio potency=57.5; Table 1). The LC50 bioassay was repeated once with similar results (ratio potency=79.4). The median lethal time (LT50) of vHaBacha100-KO-ph was $114 \pm 2.2$ h p.i., whilst that of vHaBacha100-REP-ph was $92 \pm 2.4$ h p.i. (Table 2). Survivor analysis indicated that there was a significant difference between the two viruses ($\chi^2=36.4$, $P=1.62 \times 10^{-9}$). Similar results were seen in parallel experiments; mortality resulting from infection with a ha100-null virus was delayed by 24 h ($\chi^2=29.7$, $P=5.09 \times 10^{-8}$). Therefore, the deletion of ha100 from HearNPV impacts the efficacy of the virus in killing the host insect.

DISCUSSION

ha100 is one of the uncharacterized unique genes of group II alphabaculoviruses. This gene encodes a homologue of PARG in eukaryotes. In this paper, we studied the function

Fig. 4. (a) Transfection and infection analysis results. (i–iii) Fluorescence images of transfection with recombinant bacmid bHaBacha100-KO-ph, bHaBacha100-REP-ph or bHaBac-egfp-ph in HzAM1 cells, respectively. (iv–vi) Fluorescence images of infection with vHaBacha100-KO-ph, vHaBacha100-REP-ph or vHaBac-egfp-ph at 96 h p.i., respectively. (b) Detection of HA100 expression of the recombinant viruses. Cells were infected at an m.o.i. of 5 TCID50 per cell with vHaBacha100-KO-ph (lane 1), vHaBacha100-REP-ph (lane 2), vHaBac-egfp-ph (lane 3) or HearNPV (lane 4) or were mock infected (lane 5). Cellular proteins were harvested at 72 h p.i. and separated by 10% SDS-PAGE. An anti-HA100 polyclonal antibody was used for Western blot analysis. Molecular mass markers (kDa) are shown in lane M. (c) BV one-step growth curves of recombinants viruses. HzAM1 cells were infected with vHaBacha100-KO-ph, vHaBacha100-REP-ph or vHaBac-egfp-ph. BV titres were determined at the appropriate times p.i. by an end-point dilution method. Growth curves were generated by arithmetic mean data from three infections.
of HA100 and its role in virus infectivity. Our analyses showed that the absence of ha100 did not affect BV propagation in HzAM1 cells (Fig. 4c). Transient expression assays suggested that HA100 by itself localized in the cytoplasm and shuttled into the nucleus after super-infection. In addition, deletion of ha100 did not appear to affect ODV or OB structure and morphogenesis. However, the ha100-null OBs resulted in higher LC₅₀ values and longer LT₅₀ times in third-instar H. armigera larvae, suggesting that the association of HA100 with ODVs could contribute to the virulence of HearNPV OBs. The results of this study showed that HA100 affects the oral infectivity of HearNPV in vivo but not replication of the virus in vitro.

Previous studies have shown that HA100 is associated with ODV nucleocapsid and envelope components (Deng et al., 2007), which implies that the protein would localize to the ODV in the nucleus at the late stages of infection. Transient expression of GFP-fused HA100 in HzAM1 cells resulted in cytoplasmic localization of the protein, but following HearNPV infection the localization of GFP-fused HA100 changed from the cytoplasm to the nucleus, suggesting that HearNPV provides other factor(s) to chaperone HA100 to the nucleus. In a recent study, an interaction of HA100 was identified with VP80 (HA092; Chen et al., 2001) by yeast two-hybrid and co-immunoprecipitation assays (Peng et al., 2010). The gene for VP80 is highly conserved among baculoviruses and is one of the ‘core genes’ (Herniou & Jehle, 2007). VP80 is expressed late after infection and is found to be located in infected cell nuclei associated with nucleocapsids (Müller et al., 1990; Wu et al., 2008). Using VP80 knockout viruses, it was shown to be essential for BV

Table 1. Concentration causing mortality of vHaBacha100-KO-ph and vHaBacha100-REP-ph in third-instar H. armigera larvae

<table>
<thead>
<tr>
<th>Test</th>
<th>Virus</th>
<th>LC₅₀ (OBs ml⁻¹)</th>
<th>95 % Confidence limit (OBs ml⁻¹)</th>
<th>Ratio potency (95 % confidence limit)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>1</td>
<td>HaBacha100-KO-ph</td>
<td>6.8 x 10⁵</td>
<td>4.4 x 10⁵</td>
<td>11.8 x 10⁵</td>
</tr>
<tr>
<td></td>
<td>HaBacha100-REP-ph</td>
<td>1.2 x 10⁴</td>
<td>6.0 x 10⁴</td>
<td>1.9 x 10⁴</td>
</tr>
<tr>
<td>2</td>
<td>HaBacha100-KO-ph</td>
<td>6.0 x 10⁵</td>
<td>4.0 x 10⁵</td>
<td>9.8 x 10⁵</td>
</tr>
<tr>
<td></td>
<td>HaBacha100-REP-ph</td>
<td>7.0 x 10³</td>
<td>4.0 x 10³</td>
<td>1.2 x 10⁴</td>
</tr>
</tbody>
</table>
and ODV assembly (Marek et al., 2010). VP80 also localizes in the nucleus of Bombyx mori nucleopolyhedrosis virus-infected cells (Tang et al., 2008). Further experiments will be performed to elucidate whether VP80 indeed plays a role in the transportation of HA100 into the nucleus and the contribution of HA100 in the assembly of ODVs.

Although HA100 has been identified as a structural protein of both the nucleocapsid and the envelope of ODVs (Deng et al., 2007), our results showed that ha100 is a non-essential gene for HearNPV and possibly all group II alphabaculoviruses. Moreover, growth-curve kinetics indicated that ha100 had no effect on BV infectivity (Fig. 4c). Electron microscopy showed that the ODV morphology of the knockout virus was similar to that of control viruses, suggesting that HA100 is not a major protein of ODVs, as its absence did not affect the overall structure of ODV particles and the assembly process.

Bioassay experiments demonstrated that loss of HA100 impacted on HearNPV per os infectivity. Deletion of ha100 resulted in a reduction in oral infectivity of OBs (LC50) by six- to eightfold (Table 1) but had no effect on BV production and titres (Fig. 4c). In addition, survival analysis results indicated that ha100 affects the final mortality by accelerating mortality in infected larvae by about a day. Two types of viral processes, primary midgut infection and rapid BV transmission, contribute to the virulence by per os infection. So far, several baculovirus genes have been shown to be non-essential for in vivo infection but play an important role in accelerating the mortality of the host, i.e. ac23, pe38, ac150 and ac145, or sf29. Deletion of ac23 results in infectious virus with an extended median survival time (Lung et al., 2003). The protein encoded by Autographa californica multiple nucleopolyhedrovirus (AcMNPV) pe38 promotes rapid transmission of BV to tracheolar cells and thereby enhances the virulence of infection initiated per os (Milks et al., 2003). In the case of virus infection of Heliothis virescens by AcMNPV lacking Ac150 and Ac145, the higher LC50 was due to poor establishment of primary infection (Lapointe et al., 2004). In the case of Spodoptera frugiperda multiple nucleopolyhedrovirus, deletion of sf29 reduced per os infectivity, which could be explained by an eightfold lower number of ODVs per OB (Simón et al., 2008). In our study, the results confirmed that ha100 plays a role in in vivo infectivity, but the mechanism by which ha100-null virus affects oral infection is unknown.

Previously, most investigations on PARG were carried out in higher eukaryotes. It has been reported that PARG is critical for the maintenance of steady-state poly(ADP-ribose) levels and plays important roles in modulating chromatin structure, transcription, DNA repair and apoptosis (Bonicalzi et al., 2005). However, the presence of PARG in a virus has not been reported previously. As shown in Figs 1 and 4(b), an additional band could always be detected, even in mock-infected cells. It is possible that anti-HA100 antibody might recognize the homologue of an insect PARG. The results presented here suggest that, even though HA100 appears to be a homologue of PARG, its function in insect cells in conjunction with baculovirus nucleocapsids appears to be different from that in higher eukaryotes. Further investigations on HA100 may reveal more functions of this viral PARG-like protein in addition to what is already known from research on PARG in higher eukaryotes.

### METHODS

#### Insect cells, virus and infection.
HzAM1 cells (McIntosh & Ignoffo, 1983) were maintained in Grace’s insect medium supplemented with 10% FBS (Gibco-BRL) at 28 °C and used for HA100 expression analysis. An in vivo-cloned strain of HearNPV (HaSNPV-G4; Sun et al., 1998) was used as the wild-type virus and propagated in fourth-instar H. armigera. An infectious bacmid, HaBacH28, of HearNPV has been described previously (Wang et al., 2003) and was used as a bacmid source. Viral titres were determined by an end-point dilution assay (Vlak, 1979).

#### Expression analysis of HearNPV HA100.
HzAM1 cells were infected with HearNPV at an m.o.i. of 5 TCID50 per cell. At appropriate time points p.i., cells were collected and washed with PBS. Protein samples were separated by SDS-PAGE (10 % acrylamide gel) and transferred onto nitrocellulose membrane using a semi-dry transfer cell (Bio-Rad) as recommended by the manufacturer. The blot was probed with rabbit polyclonal anti-HA100 antibody (Deng et al., 2007) followed by goat anti-rabbit IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch). The signal was detected with a BCIP/nitro blue tetrazolium kit (SABC).

#### Localization of HA100 in HzAM1 cells.
A plasmid containing the egfp-fused ha100 gene was constructed to study the localization of HA100 in HzAM1 cells. The egfp gene was digested from pEGFP-C1 (Clontech) with NheI and BamHI, and subcloned into pIZ/V5-His (Invitrogen) to generate pIZ/V5-egfp. The ha100 sequence was amplified by PCR from HearNPV genomic DNA using primers 100C fusionF (5’-GGATCCTATGGCCGAGCTTGAAATGACACCACTTAAAACAG-3’; BglII site underlined) and 100C fusionR (5’-GGCGGATCCCACACCGATGAAATGACACCGCTGTT; BamHI site underlined) and cloned into pIZ/V5-egfp to generate pIZ/V5-egfp/ha100. Plasmid DNA (5 μg) of pIZ/V5-egfp/ha100 was transfected into HzAM1 cells with Lipofectin (Invitrogen) according to the manufacturer’s protocol.

### Table 2. Time of mortality of vHaBac100-KO-ph and vHaBac100-REP-ph in third-instar H. armigera larvae

<table>
<thead>
<tr>
<th>Test</th>
<th>Virus</th>
<th>LT50 with 95% confidence interval (h p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HaBac100-KO-ph</td>
<td>114.0 (111.8–116.2)</td>
</tr>
<tr>
<td>2</td>
<td>HaBac100-REP-ph</td>
<td>92.0 (89.6–94.4)</td>
</tr>
<tr>
<td>3</td>
<td>HaBac100-KO-ph</td>
<td>114.0 (112.0–116.0)</td>
</tr>
<tr>
<td>4</td>
<td>HaBac100-REP-ph</td>
<td>90.0 (87.2–92.8)</td>
</tr>
</tbody>
</table>
Plasmid pIZ/V5-egfp was used as a control. Superinfection of HzAM1 cells was conducted with HearNPV (m.o.i. of 5 TCID₅₀ per cell) at 12 h post-transfection. At 72 h p.i., the cells were fixed (4% paraformaldehyde, 10 min) and observed in a confocal laser-scanning microscope (Leica).

Construction of the ha100-null HearNPV bacmid. The ha100 gene in the HearNPV bacmid HaBacHZ8 (Wang et al., 2003) was replaced by a gene cassette containing a chloramphenicol resistance (Cm') gene and an egfp gene controlled by the hsp70 promoter. A 2.3 kb fragment containing the Cm' and egfp genes with 57 bp upstream and downstream flanking sequences of ha100 was PCR amplified from pKS-egfp-cmr' (kindly provided by Dr M. M. van Oers, Wageningen University, The Netherlands) with primers 100delF: 5'-ATGCCGTTGAACAAAGACGCTGACATTTCAAAATTATTTTCGAGGAGACATGACATTCAAAAGGGCACCAATA-3' (nucleotides identical to the ha100 region are underlined) and transformed into Escherichia coli BW25113 containing HaBacHZ8 DNA. The ha100-null bacmid was generated by homologous recombination in E. coli, which were screened for kanamycin and chloramphenicol resistance as described previously (Fig. 3) (Hou et al., 2002). Recombinant bacmids were identified by PCR, and the correct bacmid was designated bHaBacha100-KO. As the original ph gene was disrupted during construction of the bacmid HaBacHZ8 (Wang et al., 2003), ph was reintroduced into HearNPV ha100-deletion bacmids in the ph locus to produce bHaBacha100-KO-ph (Fig. 3).

Construction of the ha100-rescued bacmid. A fragment containing ha100 under the control of its own promoter was PCR-amplified with primers REP100F (5'–GGGGATCCCATGAA-3') and REP100R (5'–GCTGACATTTCAAAATTATTTTCGAGGAGACATGACATTCAAAAGGGCACCAATA-3'), cloned into the transfer vector pFB-DUAL-ph (Song et al., 2008) and named pBF100-ph. The expression cassette of pBF100-ph was transposed into the Tn7 attachment site of bHaBacha100-KO (Wang et al., 2003). The resulting bacmid was named bHaBacha100-REP-ph (Fig. 3). bHaBac-egfp-ph containing the egfp–Cm' cassette and ph was used as a control (Fig. 3) (Song et al., 2008).

Transfection and infection assays. Bacmid DNAs of bHaBacha100-KO-ph, bHaBacha100-REP-ph and bHaBac-egfp-ph were purified from 5 ml cultures. DNA (1 µg) was used to transfect 5 × 10⁵ HzAM1 cells with the aid of Lipofectin (Invitrogen). After 120 h, the cells were centrifuged at 4000 rpm in a microfuge for 10 min. The supernatants were used to infect 6 × 10⁵ HzAM1 cells for 96 h. All the transfected and infected cells were viewed for fluorescence. Western blot analysis with infected cells was performed to detect HA100 expression. The HzAM1 cells were infected with vHaBacha100-KO-ph, vHaBacha100-REP-ph, vHaBac-egfp-ph and HearNPV at an m.o.i. of 5 TCID₅₀ per cell. Cells were collected at 72 h p.i., and cellular proteins were separated by 10% SDS-PAGE, blotted onto nitrocellulose membranes and HA100 was detected as described above.

One-step growth curves of recombinant viruses. HzAM1 cells (2 × 10⁵) were infected with vHaBacha100-KO-ph, vHaBacha100-REP-ph or vHaBac-egfp-ph at an m.o.i. of 5 TCID₅₀ per cell. Supernatants were collected at various time points (12, 24, 48, 72 and 96 h p.i.) and titres were measured by an end-point dilution assay using green fluorescence as an indicator of infection. The experiment was performed in triplicate for each virus and growth curves were generated by arithmetic mean data of three infections. The titres of the three viruses were compared with one-way analysis of variance (SPSS Inc.).

**Electron microscopy.** HzAM1 cells (1 × 10⁵) were infected with vHaBacha100-KO-ph or vHaBacha100-REP-ph at an m.o.i. of 5 TCID₅₀ per cell. Infected cells were harvested at 72 h p.i. and washed twice with PBS. The OBs of the recombinant viruses (vHaBacha100-KO-ph or vHaBacha100-REP-ph) were obtained by intrahemocoelic injection of BVs into late third-instar H. armigera larvae and purified from diseased larvae as described previously (Sun et al., 1998). All samples were processed for electron microscopy as described elsewhere (van Lent et al., 1990).

**Bioassays.** In order to determine whether the ha100 deletion had any effect on the infectivity of HearNPV, bioassays were performed as described previously (Wang et al., 2003). The OBs of recombinant viruses (vHaBacha100-KO-ph or vHaBacha100-REP-ph) were purified from separately infected H. armigera larvae. The LC₅₀ of the viruses was determined by droplet bioassay by exposing third-instar larvae to five concentrations of OBs: 1 × 10⁶, 3 × 10⁵, 1 × 10⁵, 3 × 10⁴ and 1 × 10³ OBs ml⁻¹ for 10 min and then transferred to a fresh diet. Forty-eight insects were tested per dose and the mortality was checked daily. The experiment was performed twice. The LC₅₀ values were determined by probit analysis and compared by standard lethal dose ratio comparison (SPSS Inc.).

The LT₅₀ of the viruses was determined with third-instar larvae using a food-contamination method: 10 µl OBs (1 × 10⁷ OBs ml⁻¹) of the viruses was applied to a small amount (3 mm³) of the diet of early third-instar H. armigera larvae. The intake was sufficient to kill 100% of the larvae. Time 0 was defined as the point at which larvae were transferred to the fresh diet. Forty-eight insects were used for each virus and the mortality was checked every 6 h. The LT₅₀ values of the two viruses were calculated using the Kaplan–Meier estimator and further compared using a log-rank test (Mantel–Cox).

**ACKNOWLEDGEMENTS**

This work was supported by a 973 project (2009CB118903), grants from the National Natural Science Foundation of China (30630002 and 30670078), and the PSA project from MOST and KNAW (2008A000238). We thank Dr Basil M. Arif for scientific editing of the manuscript. We also thank Dr Xiulian Sun (Wuhan Institute of Virology, CAS) for statistical analyses.

**REFERENCES**


Helicoverpa armigera

Efficient method to generate homologous recombinant baculovirus genomes in E. coli. Biotechniques 32, 783–784, 786, 788.


Engineering of baculovirus vectors for the manufacture of virion-free biopharmaceuticals. Biotechnol Bioeng n/a. (Epub ahead of print).

Characterization of five cell lines established from species of Heliothis. Appl Entomol Zool (Jpn) 18, 262–269.


Localisation of the 34 kDa polyhedron envelope protein in Spodoptera frugiperda cells infected with Autographa californica nuclear polyhedrosis virus. Arch Virol 111, 103–114.

The proteins of nonocluded Autographa californica nuclear polyhedrosis virus produced in an established cell line of Spodoptera frugiperda. J Invertebr Pathol 34, 110–118.

Cloning of biologically active genomes from a Helicoverpa armigera single-nucleocapsid nucleopolyhedrovirus isolated by using a bacterial artificial chromosome. Virus Res 97, 57–63.


Multiple nucleopolyhedrovirus 38K is a novel nucleocapsid protein that interacts with VP105, VP93, VP80, and itself. J Virol 82, 12356–12364.