Variants of open reading frame Bm126 in wild-type Bombyx mori nucleopolyhedrovirus isolates exhibit functional differences

Bifang Hao,1,2 Jinshan Huang,2 Xiulian Sun,2 Fei Deng,2 Yanfang Zhang,2 Hualin Wang,2 Hong Chen1 and Zhihong Hu2

INTRODUCTION

The family Baculoviridae comprises viruses with circular, double-stranded DNA genomes and rod-shaped, enveloped virions (Blissard et al., 2000). Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the type species and is the most widely studied of the family Baculoviridae (Ayres et al., 1994). The AcMNPV open reading frame 150 (Ac150) encodes the 11K protein with a hydrophobic N terminus and a C-terminal peritrophin-A domain (Ayres et al., 1994; Dall et al., 2001). Ac150 also encodes a cluster of charged amino acids between the hydrophobic N terminus and the C6 motif (Zhang et al., 2005). The 11K proteins are conserved among many baculovirus genomes and, in addition, homologues are found in entomopoxviruses (Dall et al., 2001). The peritrophin-A domain can be represented as CX7-18CX7CX12-14CX9-11C, where X is any amino acid residue other than cysteine, also referred to as the six-cysteine (C6) motif (Tellam et al., 1999). The C6 motif is also found in various chitinases, mucins and other proteins incorporated within the peritrophic membrane (PM), as well as in receptors and other proteins involved in cellular adhesion (Kramer & Muthukrishnan, 1997; Shen & Jacobs-Lorena, 1999; Tellam, 1996; Tellam et al., 1992). The functions of peritrophin-A are diverse but frequently involve interaction with chitin (Elvin et al., 1996; Tellam, 1996). However, it has been shown that AC150 lacks a chitin-binding activity (Lapointe et al., 2004). M2R is a stilbene-derived optical brightener known to bind to chitin and damage the PM (Wang & Granados, 2000). It was shown that M2R does not enhance the larval mortality of an Ac150 deletion mutant, indicating that AC150 may not target the PM (Zhang et al., 2005).

So far, reports on the function of Ac150 have been inconsistent. Ac150 was found as a structural component of both budded virus (BV) and occlusion-derived virus (ODV) of AcMNPV by Western blotting and immunogold electron microscopy (Lapointe et al., 2004), but others could not detect it in ODV using several approaches (Braunagel et al., 2003). Lapointe et al. (2004) reported that deletion of Ac150 alone had no effect on the oral infectivity of the virus, but in combination with Ac145, it had a host-dependent impact on oral infectivity. Zhang et al. (2005) found that when Ac150 was deleted, the

Received 2 June 2008
Accepted 31 July 2008
occlusion bodies (OBs) were significantly less virulent to larvae by oral infection, but this was not the case when ODVs were administered orally. Ohkawa (1997) found that deletion of Bm126, the Bombyx mori NPV (BmNPV) homologue of Ac150, reduced virulence of BmNPV in orally infected B. mori larvae.

In this study, the Bm126 ORF was amplified from BmNPVs isolated from five different regions of China. Sequence analyses showed that there were two subtypes of Bm126 in these isolates, Bm126-SX and Bm126-GD, and that both were different from that of the fully sequenced BmNPV T3 genotype (Gomi et al., 1999). The variation of different Bm126 subtypes occurred in the charged amino acid region between the N-terminal hydrophobic domain and the C-terminal C6 motif. Bacmid BmBacJS13 (Huang et al., 2007), originally derived from a BmNPV harbouring Bm126-SX, was used as a backbone to study the function of Bm126. The function of the different subtypes was examined by constructing a Bm126-knockout bacmid that was subsequently repaired with Bm126-SX or Bm126-GD. The results showed that, although Bm126 was not essential for viral replication, the mean survival time (ST50) of the infected larvae was significantly delayed when Bm126 was deleted. Interestingly, this delay could be rescued when the deletion mutant was repaired with Bm126-SX but not with Bm126-GD. Bm126-GD-repaired virus, however, showed a significantly higher yield of OBs, both in vitro and in vivo.

**METHODS**

**Insect cell line, larvae and virus.** BmN cells were maintained at 28 °C in TC-100 insect medium (JRH Biosciences) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL). B. mori larvae (Dazao) were reared in groups at 26 ± 1 °C on an artificial diet (Choudary et al., 1995) and third- or fifth-instar larvae were used in experiments. The wild-type (wt) BmNPV strains used in the experiments were originally isolated from the cadavers of B. mori larvae collected in Guangdong, Jiangsu, Shaanxi, Sichuan and Zhejiang provinces of China and were designated BmNPV-GD, BmNPV-JS, BmNPV-SX, BmNPV-SC and BmNPV-ZJ, respectively. BmNPV bacmids BmBacJS13 and BmBacJS13-polh were constructed previously by Huang et al. (2007) and derived from the BmNPV-SX isolate.

**Amplification of Bm126 from wt BmNPV isolates.** Viral DNAs were extracted from purified OBs of isolates BmNPV-GD, BmNPV-JS, BmNPV-SX, BmNPV-SC and BmNPV-ZJ as described by Sun & Zhang (1994) and stored at 4 °C. Using the sequence of the BmNPV T3 isolate, the primer pair 126EF (5'-CGGGATCCATGGATGTTCAAAGCCACATGATTAGAA-3') and 126DR (5'-GGCGGATCTTTAATATATATATATATATATATATATATAT-3') were designed to amplify the 600 bp downstream region of Bm126. The PCR products were gel purified and cloned into the pGEM-T Easy vector (Promega). Two to three clones from each isolate were sequenced. The deduced amino acid sequences of Bm126 were aligned using CLUSTAL_X version 1.83 and the alignment was adjusted using GeneDoc software.

**Transcriptional analysis of Bm126.** BmN cells were infected with vBmBacJS13-polh at an m.o.i. of 5 TCID₅₀ units per cell. Total RNAs were extracted with Trizol (Invitrogen), following the manufacturer’s instructions, at 0, 4, 6, 8, 12, 24, 36, 48 and 72 h post-infection (p.i.). A 3' rapid amplification of cDNA ends (3'RACE) was performed using avian myeloblastosis virus reverse transcriptase (Promega) and an oligo(dt)₁₅ three-site adaptor primer (5'-CTGATCTAGGTTAGCCGGATCCCTCTTTTTTTTTTTTTTTT3') using 1 μg purified total RNA as template to synthesize the first cDNA according to the manufacturer’s instructions. The cDNA was then used as template for PCR with primers of a three-site adaptor primer (5'-CTGATCTAGAGGTACCCGGATCC-3') and a Bm126-specific forward primer (5'-ATGTTGAACTGATCTATGATTAGAA-3'). The PCR products were analysed by agarose gel electrophoresis and the product of the 48 h p.i. sample was purified and cloned into the pGEM-T Easy vector. Three positive clones were sequenced to examine the 3' end of the Bm126 transcript.

**Construction of a Bm126 deletion bacmid, BmBacJS13.** To delete the Bm126 gene of BmBacJS13, a transfer vector containing the Bm126 flanking regions for homologous recombination was constructed in which Bm126 was replaced with the chloramphenicol resistance gene (Cm₅) under its own promoter and an egfp gene under the hsp70 (Drosophila heat shock 70) promoter. Primers 126UF (5'-GGCGGATCCCTCACAAGCGGCTTCTAGATAATG-3') and 126UR (5'-GGCGGATCCCTCACAAGCGGCTTCTAGATAATG-3') were used to amplify the 610 bp upstream flanking region of Bm126, whilst primers 126DF (5'-GGCGGATCCCTCACAAGCGGCTTCTAGATAATG-3') and 126DR (5'-GGCGGATCCCTCACAAGCGGCTTCTAGATAATG-3') were used to amplify the 600 bp downstream region of Bm126. The upstream and downstream fragments were subsequently cloned into pKS-Cm₅-egfp (kindly provided by Professor Just M. Vlak, Wageningen University, The Netherlands) and the constructed transfer vector was named pKS-126UD. pKS-126UD was digested with KpnI and Xhol to generate a linear 5.4 kb fragment containing Cm₅ and egfp in between the Bm126 flanking regions. The linear fragment was then transformed into Escherichia coli BW25113, which contains BmBacJS13 DNA (Huang et al., 2007) and helper plasmid pkD46 (Hou et al., 2002), to construct a Bm126-null bacmid. The bacmids were identified by PCR and restriction endonuclease mapping, and the correct bacmid was named BmBacα126. For the control, the egfp gene was inserted into BmBacJS13 to generate BmBacJS13-egfp.

**Construction of bacmids BmBacα126-polh, BmBacα126-SX-polh and BmBacα126-GD-polh.** The polyhedrin (polh) gene containing its own promoter from BmNPV-SX was introduced into BmBacα126 by transposition using pFastDual-polh (Huang et al., 2007) according to the Bac-to-Bac system manual (Invitrogen). pFastDual-polh was derived from pFastBacDual (Invitrogen), in which the promoters of AcMNPV p10 and polh were replaced by the BmNPV polh gene (Huang et al., 2007). The generated bacmid was designated BmBacα126-polh (see Fig. 3a). To compare the properties of Bm126 from the different isolates, the promoter and coding sequences were amplified from BmNPV-SX and BmNPV-GD using primers 126RF (5'-GGCGGATCCCTCACAAGCGGCTTCTAGATAATG-3') and 126RR (5'-GGCGGATCCCTCACAAGCGGCTTCTAGATAATG-3'); BamHI site underlined) and 126RF (5'-GGCGGATCCCTCACAAGCGGCTTCTAGATAATG-3'); EcoRI site underlined) were designed to amplify the coding region of Bm126. The PCR products were gel purified and cloned into the pGEM-T Easy vector (Promega). Two to three clones from each isolate were sequenced. The deduced amino acid sequences of Bm126 were aligned using CLUSTAL_X version 1.83 and the alignment was adjusted using GeneDoc software.

**Transfection and infection of BmN cells.** BmN cells (6 × 10⁴) were transfected with 2.0 μg of the appropriate bacmid DNA (BmBacJS13-polh, BmBacJS13-egfp, BmBacα126-polh, BmBacα126-SX-polh or
BmBac\textsubscript{126-GD-polh} using 6 \textmu l lipofectin (Invitrogen Life Technologies). The cells were incubated for 4 h after transfection and replenished with 2 ml fresh medium supplemented with 10% FBS, penicillin (100 \textmu g ml\textsuperscript{-1}) and streptomycin (30 \textmu g ml\textsuperscript{-1}). Virus-containing supernatant was collected from the transfected BmN cells at 96 h post-transfection (p.t.) and used to infect BmN cells. The cells were observed by light and fluorescent microscopy at 48 h p.i.

**Comparison of BV one-step growth curves of recombinant viruses.** BmN cells were infected with BmBacJS13-polh, BmBac\textsubscript{126-polh}, BmBac\textsubscript{126-SX-polh} or BmBac\textsubscript{126-GD-polh} at an m.o.i. of 5 TCID\textsubscript{50} per cell. After 1 h of incubation, the virus-containing medium was removed. The cells were washed three times with serum-free medium, and fresh medium containing FBS was added. In all experiments, time 0 was defined as the point at which fresh medium was added. At the appropriate time points (0, 12, 24, 48, 72 and 96 h p.i.), supernatants were collected and the titres of the BV were determined using an end-point dilution assay (EPDA). OBs were used as a marker of infection during the assay. Each viral infection was carried out in triplicate and the experiment was repeated once. BV titres at different times p.i. were pooled and log-transformed for further comparison using a log-rank test (Snedecor, 1989).

**In vivo bioassay.** OBs of the recombinants were purified from fifth-instar B. mori larvae infected with vBmBacJS13-polh, vBmBac\textsubscript{126-polh}, vBmBac\textsubscript{126-SX-polh} or vBmBac\textsubscript{126-GD-polh}. The median lethal concentrations (LC\textsubscript{50}) of the viruses were determined by feeding third-instar larvae an artificial diet containing five different concentrations of OBs. Fifty larvae were used per concentration of each virus and experiments were repeated twice. The mortality of the infected larvae was observed daily. LC\textsubscript{50} values and their 95% confidence limits were determined by probit analysis (SPSS). The LC\textsubscript{50} values of the viruses were compared further using a two-sided z-test (Snedecor, 1989).

The median survival time (ST\textsubscript{50}) of the viruses was determined for third-instar larvae using a food-contamination method. OBs (4 \textmu l 5 \times 10\textsuperscript{4} OBs ml\textsuperscript{-1}) of the viruses were applied to a small plug of artificial diet in individual containers, and only larvae that completely ingested the contaminated diet within 5 h were used in the experiments (n>40). Time 0 was defined as the point at which the larvae were placed on the contaminated diet and mortality was recorded at intervals of 4 h. ST\textsubscript{50} values of the viruses were calculated using the Kaplan–Meier estimator and further compared using a log-rank test (Kalbfleisch & Ross, 1980). The experiments were repeated twice.

**Assay for OB production in vitro or in vivo.** BmN cells (1 \times 10\textsuperscript{5}) were infected with vBmBacJS13-polh, vBmBac\textsubscript{126-polh}, vBmBac\textsubscript{126-SX-polh} or vBmBac\textsubscript{126-GD-polh} at an m.o.i. of 5. At 48 h p.i., infected cells and medium were harvested completely and centrifuged at 4000 g for 10 min to pellet the cells and OBs. Part of the cell pellets were lysed using a supersonic disrupter to count the OBs using a haemocytometer. The number of OBs in cells was compared using one-way ANOVA with virus as a factor after the data had been log-transformed. If significant effects were found, Fisher’s least significant difference (LSD) test was used to separate the means. The sequence of the \textit{vBmBacJS13} is shown in Fig. 2(b). Two early promoter ORFs were amplified by PCR from BmNPV-GD, BmNPV-JS, BmNPV-SX and BmNPV-ZJ. The PCR products were cloned and sequenced. The sequences from any one isolate were the same, but the sequences from different isolates showed there were two subtypes of \textit{Bm126}. BmNPV-JS, BmNPV-SX and BmNPV-ZJ shared one subtype (\textit{Bm126-SX}), whilst BmNPV-GD and BmNPV-SX shared another subtype (\textit{Bm126-GD}), and both were different from that of \textit{BmNPV T3} (\textit{Bm126-T3}). The deduced amino acid sequences of BM126s and AC150 are presented in Fig. 1. All sequences contained a variable region flanked by a hydrophobic N terminus and a C6 motif. The Arg-Gly-Asp (RGD) motif in AC150 was conserved in all sequences. Bm126-GD, Bm126-SX and Bm126-GD-polh, Bm126-SX-polh or BmBac\textsubscript{126-GD-polh} by oral feeding with OBs at the LC\textsubscript{99} concentration (99% lethal concentration). Twenty-four hours later, only larvae that had completely ingested the contaminated diet were transfer to a fresh diet. Larvae were kept at 27 °C and observed daily. Only those that died after 3 days p.i. were collected. The collected cadavers were kept at room temperature until completely liquefied, weighed and homogenized, and the OBs were counted with a haemocytometer as outlined by Hunter-Fujita (1998). For each virus treatment, 30 cadavers were used. The yield of OBs per cadaver killed by each of the four viruses was compared by one-way ANOVA after the data had been log-transformed. If significant effects were found, Fisher’s LSD test was used to separate the means.

**RESULTS**

**Sequence analysis of Bm126**

The different \textit{Bm126} ORFs were amplified by PCR from BmNPV-GD, BmNPV-JS, BmNPV-SX, BmNPV-SC and BmNPV-ZJ. The PCR products were cloned and sequenced. The sequences from any one isolate were the same, but the sequences from different isolates showed there were two subtypes of \textit{Bm126}. BmNPV-JS, BmNPV-SX and BmNPV-ZJ shared one subtype (\textit{Bm126-SX}), whilst BmNPV-GD and BmNPV-SX shared another subtype (\textit{Bm126-GD}), and both were different from that of \textit{BmNPV T3} (\textit{Bm126-T3}). The deduced amino acid sequences of BM126s and AC150 are presented in Fig. 1. All sequences contained a variable region flanked by a hydrophobic N terminus and a C6 motif. The Arg-Gly-Asp (RGD) motif in AC150 was conserved in all sequences. Bm126-GD, Bm126-SX and Bm126-GD-polh, Bm126-SX-polh or BmBac\textsubscript{126-GD-polh} by oral feeding with OBs at the LC\textsubscript{99} concentration (99% lethal concentration). Twenty-four hours later, only larvae that had completely ingested the contaminated diet were transferred to a fresh diet. Larvae were kept at 27 °C and observed daily. Only those that died after 3 days p.i. were collected. The collected cadavers were kept at room temperature until completely liquefied, weighed and homogenized, and the OBs were counted with a haemocytometer as outlined by Hunter-Fujita (1998). For each virus treatment, 30 cadavers were used. The yield of OBs per cadaver killed by each of the four viruses was compared by one-way ANOVA after the data had been log-transformed. If significant effects were found, Fisher’s LSD test was used to separate the means.

**Transcriptional mapping of the 3’ ends of Bm126**

The timing of \textit{Bm126} transcription and the 3’ end of the \textit{Bm126} transcripts were determined using 3’RACE. As shown in Fig. 2(a), an RT-PCR product of 360 bp was first detected at 6 h p.i. and this band continued to be detected until 72 h p.i. The sequence of the \textit{Bm126} region of BmBacJS13 is shown in Fig. 2(b). Two early promoter motifs, CAGT and a TATA box-like sequence, were observed at 166 and 96 nt upstream of the translation start codon ATG (Fig. 2b), whilst a baculovirus late promoter TAAG motif was identified 134 nt upstream of the ATG. Whether these early and late motifs are used for \textit{Bm126} transcription needs to be analysed further.

Three clones containing the fragment of the 48 h p.i. RT-PCR product were sequenced. All of the sequences were
identical and the poly(A) tails were found immediately after the stop codon of Bm126 (Fig. 2b). There were two poly(A) signals (AATAAA) located upstream of the stop codon of Bm126 (Fig. 2b). The 3' RACE results suggested that they may be used as the poly(A) signal for the Bm126 transcript.

**Construction and identification of Bm126-null and Bm126-SX- or Bm126-GD-repaired bacmids**

To examine the role of the Bm126 gene in the viral infection cycle, Bm126 was knocked out from bacmid BmBacJS13 (derived from BmNPV-SX) (Huang et al., 2007) by homologous recombination in *E. coli* (Hou et al., 2002) (Fig. 3a). The resulting bacmid (BmBacA126) was selected by kanamycin and chloramphenicol resistance and confirmed by PCR (data not shown) and restriction endonuclease analysis. As shown in Fig. 3(b, lanes 1 and 2), XbaI digestion analysis revealed that the 19 kb C fragment (which contained Bm126) present in BmBacJS13 was replaced by the 21 kb C' fragment in BmBacA126. This change was due to the substitution of Bm126 by CmR8 and egfp. The restriction profiles indicated that the deletion mutant was constructed correctly.

To test whether Bm126 had any effect on oral infection, polh and Bm126-SX, or polh and Bm126-GD, were reintroduced into BmBacA126 by transposition. The resulting bacmids, BmBacA126-polh, BmBacA126-SX-polh and BmBacA126-GD-polh (Fig. 3a), were selected by PCR (data not shown) and digested with various restriction endonucleases. Fig. 3(b) shows the XbaI digestion profiles of the recombinant and control viruses. In BmBacA126-polh and BmBacJS13-polh, where the polh gene was introduced, the XbaI H fragment of BmBacJS13 (4.9 kb) was substituted by a 7.0 kb H' fragment (Fig. 3b, lanes 3 and 4). In BmBacA126-SX-polh and BmBacA126-GD-polh, where the polh and Bm126-SX or Bm126-GD gene were introduced, the H fragment was substituted by a 7.5 kb H'' fragment (Fig. 3b, lanes 5 and 6). All of the changes were as expected and the results indicated that all of the recombinant bacmids were constructed correctly.

**Recombinant virus replication in BmN cells and one-step growth curves of BV production**

BmN cells were transfected with BmBacJS13-polh, BmBacJS13-egfp, BmBacA126-polh, BmBacA126-SX-polh or BmBacA126-GD-polh. At 36 h p.t., fluorescence was observed in all of transfected cells except for those with BmBacJS13-polh, which did not harbour the egfp gene (data not shown). The supernatants of transfected cells were harvested at 96 h p.t. and used to infect healthy BmN cells. At 48 h p.i., fluorescence was observed in all transfected cells except for those with BmBacJS13-polh, whilst OBs and other cytopathic effects could be observed in all of the samples (Fig. 4a), indicating that Bm126 is not essential for virus replication.

To analyse BV yields of the recombinant viruses, one-step BV growth curves were determined by EPDA. The results (Fig. 4b) showed that all of the recombinant viruses had similar dynamics of BV production. Statistical analysis indicated that there was no significant difference among the titres of the four viruses at different times (*F*=0.329, d.f.=3, 112, *P*=0.804).

**Bioassay analyses**

To determine the LC50 of the recombinant viruses, third-instar larvae were infected with five different concentrations of OBs of vBmBacJS13-polh, vBmBacA126-polh, vBmBacA126-SX-polh or vBmBacA126-GD-polh. The results are presented in Table 1. Statistical analysis indicated that there was no significant difference in the LC50 value of vBmBacA126-polh from that of vBmBacJS13-polh (*Z*=0.007, *P*>0.05), vBmBacA126-SX-polh (*Z*=0.47, *P*>0.05) or vBmBacA126-GD-polh (*Z*=0.307, *P*>0.05). Therefore, deletion of Bm126 did not affect the LC50 of the virus.
ST₅₀ values of the recombinant viruses were determined by orally feeding third-instar B. mori using a food-contamination method (Table 1). The ST₅₀ of vBmBacJS13-polh was 116 ± 0.62 h, whilst that of vBmBacΔ126-polh was 128 ± 1.30 h. Analysis revealed a significant difference in the ST₅₀ of vBmBacΔ126-polh and vBmBacJS13-polh (χ² = 19.95, P = 7.94 × 10⁻⁶), indicating that deletion of Bm126 resulted in a significant delay of ST₅₀. When Bm126-SX was reintroduced into the genome, the resulting vBmBacΔ126-SX-polh had a similar ST₅₀ to that of vBmBacJS13-polh (χ² = 0.89, P = 0.35), suggesting that delay of ST₅₀ was due solely to the deletion of Bm126 and that a repair rescue was possible. However, when Bm126-GD was reintroduced into the Bm126 deletion mutant, the resulting vBmBacΔ126-GD-polh had an ST₅₀ (128 ± 1.33 h) similar to that of the Bm126 deletion mutant (Table 1). Statistical analysis (using a log-rank test) indicated that there were significant differences in the ST₅₀ values of vBmBacΔ126-
GD-polh and vBmBacΔ126- SX-polh ($\chi^2 = 43.12, P = 5.14 \times 10^{-11}$) and of vBmBacJS13-polh ($\chi^2 = 35.92, P = 2.05 \times 10^{-7}$). Therefore, Bm126-GD was unable to repair the ST50 delay caused by Bm126 deletion.

Table 1. Bioassays of the recombinant viruses in third-instar B. mori larvae

<table>
<thead>
<tr>
<th>Virus</th>
<th>LC$_{50}$ ± SEM (OBs ml$^{-1}$)</th>
<th>Slope ± SEM</th>
<th>$\chi^2$/d.f.</th>
<th>ST$_{50}$ ± SEM (h)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>vBmBacJS13-polh</td>
<td>$3.31 \pm 1.4 \times 10^5$</td>
<td>$1.55 \pm 0.17$</td>
<td>11.1/4</td>
<td>$116 \pm 0.62$</td>
</tr>
<tr>
<td>vBmBacΔ126-polh</td>
<td>$3.29 \pm 0.9 \times 10^5$</td>
<td>$1.83 \pm 0.21$</td>
<td>33.1/4</td>
<td>$128 \pm 1.30$</td>
</tr>
<tr>
<td>vBmBacΔ126-SX-polh</td>
<td>$5.08 \pm 1.6 \times 10^5$</td>
<td>$1.80 \pm 0.19$</td>
<td>8.66/4</td>
<td>$116 \pm 1.62$</td>
</tr>
<tr>
<td>vBmBacΔ126-GD-polh</td>
<td>$3.15 \pm 1.3 \times 10^5$</td>
<td>$2.14 \pm 0.25$</td>
<td>14.2/4</td>
<td>$128 \pm 1.33$</td>
</tr>
</tbody>
</table>

*ST$_{50}$ values were determined in third-instar larvae at a concentration of $5 \times 10^8$ OBs ml$^{-1}$. The mortality of the dose was 100%.
observed that the cells infected by BmBacΔ126-GD-polh appeared to produce more OBs than the others (data not shown). The number of OBs was then counted as described in Methods. The results showed that vBmBacΔ126-GD-polh yielded the highest number of OBs (Table 2). Statistical analysis indicated there were significant differences between vBmBacΔ126-GD-polh and the other viruses ($F=27.418$, d.f.=$3$, $476$, $P=1.46 \times 10^{-4}$).

The levels of POLH expression were examined using standard SDS-PAGE and Western blotting. When total proteins from same number of infected cells were loaded on the gel, the expression level of POLH from vBmBacΔ126-GD-polh-infected cells appeared to be much higher than that of the cells infected by the other viruses (Fig. 5a). Western blot results also showed that levels of POLH expression were indeed higher in vBmBacΔ126-GD-polh-infected BmN cells (Fig. 5b), whereas VP39 expression was similar among the cells infected with vBmBacJS13-polh, vBmBacΔ126-polh, vBmBacΔ126-SX-polh or vBmBacΔ126-GD-polh (Fig. 5b).

The production of OBs in the larvae was also investigated. As shown in Table 2, the number of OBs produced by BmBacΔ126-GD-polh-infected larvae was significantly higher than that of the other viruses (vBmBacJS13-polh, $P=8.3 \times 10^{-4}$; vBmBacΔ126-polh, $P=5.8 \times 10^{-6}$ and vBmBacΔ126-SX-polh, $P=5.0 \times 10^{-4}$). In summary, vBmBacΔ126-GD-polh produced significantly higher levels of OBs both in vitro and in vivo in comparison with the other recombinant viruses.

**DISCUSSION**

In this report, Bm126 was amplified from five different Chinese isolates of BmNPV. Sequence analyses showed that the isolates were composed of two subtypes of Bm126: Bm126-SX and Bm126-GD. Including the previously reported Bm126-T3 (Gomi et al., 1999), it is evident that there exist at least three subtypes of Bm126 in natural BmNPV isolates. It has been reported that there is one or more copies of the 11K protein in baculovirus; for example, there are five copies of the 11K gene in Xestia c-nigrum granulovirus (Dall et al., 2001). This is the first evidence that different subtypes of Bm126 exist in natural baculovirus isolates. As our study showed that different subtypes of Bm126 may have different properties under certain conditions, the multiple copies or different subtypes of the 11K genes may have evolved to fine-tune the genes’ function under different host or tissue environments.

The 3’RACE results showed that Bm126 was transcribed from early to late stages of infection (Fig. 2a). This result is consistent with the observation that both early and late promoters exist in the upstream region of Bm126 (Fig. 2b). Microarray analysis also showed that Bm126 had a transcription pattern similar to that of gP64 (Iwanaga et al. 2004), which is known to be a gene under the control of both early and late promoters.

Deletion of Bm126 did not affect BV infectivity in BmN cells (Fig. 4a, b), indicating that Bm126 is not an essential gene for virus replication. This is consistent with the results of Ohkawa (1997) who showed that the growth curve of a Bm126 deletion mutant was indistinguishable from that of the wild-type virus. Deletion of Bm126 resulted in successful oral infection but with a delayed ST50, and this delay was rescued in a repaired virus. Ohkawa (1997) also reported that Bm126-deleted virus took about 1 day longer to kill orally inoculated first-instar larvae compared with BmNPV T3-infected larvae. These results are in agreement

---

**Table 2. OB yields in BmN cells and in B. mori larvae**

<table>
<thead>
<tr>
<th>Virus</th>
<th>OB yield (mean ± SEM)</th>
<th>BmN cells (×10⁶)*†</th>
<th>Larva‡†</th>
</tr>
</thead>
<tbody>
<tr>
<td>vBmBacJS13-polh</td>
<td>1.77 ± 0.11 × 10⁷ a</td>
<td>6.03 ± 0.32 × 10⁷ a</td>
<td></td>
</tr>
<tr>
<td>vBmBacΔ126-polh</td>
<td>1.49 ± 0.11 × 10⁷ a</td>
<td>5.44 ± 0.43 × 10⁷ a</td>
<td></td>
</tr>
<tr>
<td>vBmBacΔ126-SX-polh</td>
<td>1.48 ± 0.06 × 10⁷ a</td>
<td>5.98 ± 0.37 × 10⁷ a</td>
<td></td>
</tr>
<tr>
<td>vBmBacΔ126-GD-polh</td>
<td>2.73 ± 0.08 × 10⁷ b</td>
<td>8.45 ± 0.51 × 10⁷ b</td>
<td></td>
</tr>
</tbody>
</table>

*BmN cells infected with virus at an m.o.i. of 5 TCID₅₀ per cell.
†Viruses with different letters (a vs b) in the same column have significantly different means ($P<0.05$, one-way ANOVA) in pairwise comparisons between viruses.
‡OBs produced in larvae products were determined in third-instar larvae using the LC₉₉ concentration.
with previous findings that AC150 is a *per os* infectivity factor but is not essential for oral infection (Lapointe *et al.*, 2004; Zhang *et al.*, 2005). It has been shown that the deletion of *Bm126* and *Ac150* results in reduced foci of primary infection (Ohkawa, 1997; Zhang *et al.*, 2005). Therefore, it is possible that the reduced foci of primary infection resulted in delayed ST50 in the larvae infected with a *Bm126*-null BmNPV.

Different phenotypes were observed when different sub-types of *Bm126* were used to repair a *Bm126*-null virus. The delay of ST50 caused by deletion of *Bm126-SX* was rescued when repaired with *Bm126-SX* but not with *Bm126-GD*. However, the *Bm126-GD*-repaired virus showed a significant increase in OB production, both *in vitro* and *in vivo*. The BV growth curve of the *Bm126-GD*-repaired virus was not significantly different from that of the others (Fig. 4b). Therefore, the increase in OB production is unlikely to be due to an increase in BV production. The major difference between *Bm126-GD* and *Bm126-SX* is that the former contains an RGDDKD sequence (including an RGD motif) and lacks a short stretch of six ND repeats. The RGD motif of many proteins has been shown to interact with subsets of host cell-surface integrins that are critical for a variety of cell functions (Hynes, 1992) such as regulation of gene expression, activation of focal adhesion kinases, activation of cytoskeleton elements, endocytosis, attachment, motility, cell survival, cell-cycle progression, cell growth, apoptosis and differentiation (Giancotti & Ruoslahti, 1999; Ruoslahti & Pierschbacher, 1987). In our experiments, cells infected with *Bm126-GD*-repaired virus showed a different phenotype; for example, the infected cells remained attached to the surface of the flask and survived for longer compared with the cells infected by other viruses, which became detached from the surface due to infection (data not shown). We suspect that the RGD motif in *Bm126-GD* might activate an integrin-mediated signalling pathway, which causes the upregulation of gene transcription and cell survival, and results in increased OB yield. Whether this assumption is true needs further investigation. In the Western blot results (Fig. 5b), expression of the major nucleocapsid protein, VP39, of *Bm126-GD*-repaired virus was not obviously different from that of the other viruses. However, we cannot exclude the possibility that there was a slight increase in VP39 expression that was not detected by Western blot analysis. Our unpublished data showed that there is no significant difference in OB production between wild-type BmNPV-GD and BmNPV-SX. However, it should be noted that there are significant differences in the restriction enzyme profiles of the genomes of wild-type BmNPV-GD and BmNPV-SX, indicating that there are other differences in their genome in addition to *Bm126*. It was suggested that the RGD motif in *Ac150* might play a role in *per os* infectivity (Lapointe *et al.*, 2004; Zhang *et al.*, 2005). However, *Bm126-SX*, which has been shown to be a *per os* infectivity factor, does not contain an RGD motif, whilst *Bm126-GD*, which contains an RGD motif, did not appear to influence oral infection. Therefore, it is likely that the function as a *per os* infectivity factor of AC150 homologues is not related to the RGD motif, as least in the case of *Bm126*. The exact function of *Bm126* and its homologues remains to be investigated further.

**ACKNOWLEDGEMENTS**

This work was supported financially by the following grants: 973 (2003CB114202), the National Natural Science Foundation of China project (30630002) and 863 project (2006AA10A210), and the Programme Strategic Scientific Alliances between China and The Netherlands (2004CB720404). We thank Dr Basil Arif for scientific editing of the manuscript and Professor Cheng Lu of the South-west University for providing *B. mori* eggs.

**REFERENCES**


