Bombyx mori nucleopolyhedrovirus SNF2 global transactivator homologue (Bm33) enhances viral pathogenicity in B. mori larvae

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The SNF2 global transactivator gene homologue (Bm33) of Bombyx mori nucleopolyhedrovirus (BmNPV) is one of the genes exclusive to group I NPVs, but its function remains unknown. This study describes the characterization of Bm33. Transcriptional analysis suggested that Bm33 is an early gene, as its transcript was observed at 4 h post-infection in BmNPV-infected BmN cells. To examine the role of Bm33 during BmNPV infection, a Bm33 deletion mutant (BmORF33D) was constructed and its infectivity was characterized in BmN cells and B. mori larvae. BmORF33D did not have any obvious defects in the production of budded viruses (BVs) or occlusion bodies (OBs) in BmN cells compared with wild-type BmNPV. Larval bioassays revealed that deletion of Bm33 did not reduce virus infectivity. However, BmORF33D took approximately 10–15 h longer than wild-type BmNPV to kill B. mori larvae when tested by either BV injection or OB ingestion. These results suggest that Bm33 is not essential for virus growth in vitro or in vivo, but that it accelerates the time of death of B. mori larvae.

INTRODUCTION

The family Baculoviridae comprises a diverse group of arthropod-specific DNA viruses. They have been reported worldwide, mostly from insects of the order Lepidoptera, but also from the orders Diptera and Hymenoptera (Federici, 1997). Nucleopolyhedroviruses (NPVs) form a genus in the family Baculoviridae and have a large, circular, double-stranded DNA genome within a rod-shaped virion. NPVs produce two types of virion during the infection cycle; an occlusion-derived virus (ODV), which transmits infection from insect to insect (oral infection) and is found in occlusion bodies (OBs), and a budded virus (BV), which spreads infection to neighbouring cells (Federici, 1997).

The SNF2 protein is an ATPase that acts as the catalytic subunit of the multi-subunit SWI/SNF complex (Becker & Horz, 2002). The SNF2 family of enzymes are related in sequence to DExx box helicases, but they do not possess helicase activity. Recent biochemical and structural studies suggest that the mechanism by which these enzymes act involves ATP-dependent translocation on DNA. Genome sequencing has revealed significant breadth and diversity within the SNF2 family with 17 genes for SNF2 family proteins in Saccharomyces cerevisiae and at least 32 in humans (Flaus et al., 2006).

Sequence comparison of baculovirus genomes has shown that group I NPVs, but not group II NPVs (granuloviruses), encode homologues of the SNF2 global transactivator family (Ikeda et al., 2006); however, to date, their functions remain unclear. In this study, we examined the role of Bombyx mori NPV (BmNPV) orf33 [Bm33, a homologue of Autographa californica multiple NPV (AcMNPV) ac42], a gene encoding the SNF2 family protein homologue. By constructing a Bm33 deletion BmNPV, we showed that Bm33 is not essential for virus growth in vitro or in vivo, but that it may act as a virulence factor in B. mori larvae.

METHODS

Insects, cells and viruses. B. mori larvae (F1 hybrid of Kinshu × Showa) were reared as described previously (Katsuma et al., 2006). The cell line BmN was maintained in TC-100 medium with 10% fetal bovine serum as described by Katsuma et al. (2006). The BmNPV T3 isolate (Gomi et al., 1999) was propagated in BmN cells. Virus titres were determined by plaque assay as described previously (Katsuma et al., 2006). BmN cells were infected with BmNPVs at an m.o.i. of 5.

Construction of the Bm33 deletion mutant by insertion of a β-galactosidase gene cassette. To construct a plasmid for deletion of Bm33, a 5.3 kb fragment (nt 29558–34848; GenBank accession no. L33180) was purified from the BmNPV T3 genomic clone (Maeda & Majima, 1990) and inserted into pTZ19B. The plasmid was digested with BstXI, and ligated to a β-galactosidase gene cassette containing a Drosophila melanogaster heat-shock protein promoter (hsp70-LacZ...
cassette; Kamiita et al., 1993). The resultant plasmid was co-transfected with wild-type (wt) BmNPV DNA into BmN cells using Cellfectin (Invitrogen). A Bm33 deletion BmNPV (BmORF33D) was isolated by identification of plaques expressing β-galactosidase (Katsuma et al., 2006). Deletion of Bm33 was confirmed by PCR using the primers shown in Supplementary Table S1 (available in JGV Online).

**Construction of Bm33-null BmNPV.** To generate a Bm33-null mutant, we constructed a plasmid containing the flanking region of Bm33. DNA fragments containing both upstream (nt 29071–30151) and downstream (nt 31673–32750) regions of Bm33 were amplified by PCR using various primers (see Supplementary Table S1) and cloned into pcDNA3.1(–) (Invitrogen) and were designated orf33-null pcDNA. BmN cells were co-transfected with BmORF33D DNA and orf33null pcDNA using Cellfectin. A Bm33-null BmNPV (BmORF33null) was isolated by identification of white plaques that did not express β-galactosidase (Katsuma et al., 2006). Complete deletion of Bm33 was confirmed by PCR using the primers shown in Supplementary Table S1.

**Construction of BmNPV expressing His-tagged Bm33.** To generate BmNPV expressing His-tagged Bm33, we amplified a His-tagged Bm33 fragment by PCR using the primers shown in Supplementary Table S1 and cloned it into orf33null pcDNA. The resultant plasmid was co-transfected with BmORF33D DNA into BmN cells using Cellfectin. BmNPV expressing His-tagged Bm33 (BmORF33His) was isolated by identification of plaques that did not express β-galactosidase (Katsuma et al., 2006). Insertion of His-tagged Bm33 was confirmed by PCR.

**Assays for BV production.** To determine virus growth curves, BmN cells were infected with BmNPV T3 or BmORF33D at an m.o.i. of 5. After 1 h of incubation, virus-containing culture medium was removed and fresh medium was added after two washes with serum-free TC-100 medium [0 h post-infection (p.i.)]. A small amount of culture medium was harvested at specific time points. BV production was determined by plaque assay.

**Western blotting.** BmN cells were infected with BmORF33His at an m.o.i. of 5 and harvested at 12, 24 and 48 h p.i. Biochemical fractionation of BmN cells was performed as described previously (Jarvis et al., 1991). SDS-PAGE and Western blotting were performed using anti-His antibody (Qiagen) as described previously (Katsuma et al., 2007).

**RT-PCR.** Total RNA was reverse transcribed, diluted and used for PCR as described previously (Katsuma et al., 2006). The primers for PCR are shown in Supplementary Table S1. Real-time PCR experiments were performed as described previously (Iwanaga et al., 2004).

**Rapid amplification of cDNA ends (RACE).** We performed 5′-RACE experiments to determine the transcription start site of Bm33. For 5′-RACE, we used a GeneRacer kit (Invitrogen) as described previously (Katsuma et al., 2004). Total RNA was isolated by Trizol reagent (Invitrogen) from BmNPV-infected BmN cells (at an m.o.i. of 5) at 4 or 12 h p.i., and first-strand cDNA was synthesized from 5 μg total RNA. The sequences of the Bm33-specific PCR primers are listed in Supplementary Table S1. Amplified RACE fragments were cloned into the vector pGEM-T Easy (Promega) and DNA sequences were determined using an ABI Prism 3100 DNA sequencer (Applied Biosystems).

**Alignment and phylogenetic analysis.** Amino acid sequences were aligned using the CLUSTAL W program (Thompson et al., 1994). Distances between the amino acid sequences were calculated according to the Jones–Taylor–Thornton matrix with PRODIST in the PHYLIP 3.65 package (Felsenstein, 1989). A neighbour-joining tree was constructed with the NEIGHBOR program in PHYLIP. The reliability of the tree was tested by bootstrap analysis with 1000 replications.

**Larval bioassays.** The LD_{50} of BV was determined in fifth-instar larvae by haemocoeic injection with different doses of BV diluted in TC-100 medium. The median lethal concentration (LC_{50}) of OBs was determined in third-instar larvae by feeding them different concentrations of OBs diluted in ddH_{2}O. The median lethal time (LT_{50} time in h required to kill half of the population) was determined either by haemocoeic injection of BV into fifth-instar larvae or by feeding third-instar larvae with OBs. *B. mori* larvae were inoculated with BV by haemocoeic injection within 8 h of moulting to the fifth instar. To determine the LC_{50} or LT_{50} of OBs, larvae were inoculated orally within 8 h of moulting to the third instar. In all cases, third-instar larvae inoculated with 1.3 × 10^6 OBs of BmNPV T3, BmORF33D or BmORF33null ml⁻¹ led to 100% mortality. OBs were produced in fifth-instar *B. mori* larvae, purified by centrifugation and resuspended in ddH_{2}O. Purified OBs were diluted in ddH_{2}O and quantified using a haemocytometer. Third-instar larvae (within 8 h of moulting) were inoculated orally by exposing them to a 2800 mm² area of artificial diet that was surface-contaminated with OBs (Kamita et al., 2005). At least 20 larvae per dose were used in each of the two independent experiments.

**RESULTS**

**Members of the SNF2 global transactivator family encoded by group I NPVs**

All group I NPV genomes sequenced to date encode the SNF2 family of global transactivators (Ikeda et al., 2006). The SNF2 homologues from group I NPVs share some similarity with domains of the SW1/SNF complex, and they are smaller than similar proteins in *D. melanogaster* or yeast (Hughes & Friedman, 2003). However, the domain structures of baculovirus SNF2 proteins are conserved with a large central SNF2-like ATPase domain and a helicase C terminus (Fig. 1a). Phylogenetic analysis using both domains of baculoviral and animal SNF2s showed that the SNF2 homologues encoded by BmNPV, AcMNPV and *Rachiplusia ou* NPV (RoMNPV) formed different branches from those encoded by other group I NPVs (Fig. 1b).

**Transcriptional analysis of Bm33**

Expression of Bm33 was investigated by real-time RT-PCR analysis at various times after BmNPV infection. As shown in Fig. 2(a), a Bm33 transcript was detected at 4 h p.i., suggesting that Bm33 is a baculovirus early gene. This transcript continued to increase up to 24 h p.i. (Fig. 2a). In the upstream region of the Bm33 translation start codon, two consensus sequences of the baculovirus early gene promoters (CAGT) were found (−110 to −107 and −137 to −134, Fig. 2b). The Bm33 transcription start site was determined by 5′-RACE using a gene-specific primer. cDNA clones were successfully isolated from total RNA at 12 h p.i. but not at 4 h p.i., supporting the RT-PCR result that Bm33 is expressed at a lower level at 4 h p.i. DNA sequence analysis of 11 cloned fragments revealed that the
Major transcription start site of Bm33 was 42 nt upstream of the translation start codon, and was located within CAAT (Fig. 2b). This sequence may be a variation of the insect RNA polymerase II consensus start-site sequence CAGT (Pullen & Friesen, 1995).

### Expression of Bm33

To examine localization of the Bm33 protein in BmN cells, we generated a mutant BmNPV expressing His-tagged Bm33 (BmORF33His, Fig. 3a). BmN cells were infected with BmORF33His at an m.o.i. of 5, and nuclear and cytosolic fractions of infected cells, prepared at 12, 24 and 48 h p.i., were subjected to Western blot analysis using anti-His antibody. The expression of Bm33 in BmN cells was not detected at any of these periods. However, RT-PCR analysis showed that Bm33 was expressed in BmORF33His-infected BmN cells, suggesting that His-tagged Bm33 was driven by its natural promoter (data not shown). Two possible explanations for these observations are: (i) promoter activity was so low that the expression of Bm33 was not detected by Western blot analysis, or (ii) the His-tag epitope fused to the Bm33 C terminus could not be detected due to low sensitivity of the anti-His antibody.

### Construction of a mutant BmNPV lacking functional Bm33

To determine the role of Bm33 during viral infection in BmN cells and B. mori larvae, we constructed a plasmid with the β-galactosidase gene under the control of the Drosophila heat-shock protein promoter inserted within the Bm33 coding region such that the portion of the Bm33 coding region (~550 bp) that encodes the N-terminal region (DEAD/DEAH box domain) of Bm33 was deleted (Figs 1a and 3a). We then generated a Bm33-deficient BmNPV by homologous recombination in BmN cells. To do this, the plasmid DNA was co-transfected with BmNPV DNA into BmN cells and a recombinant virus (BmORF33D) was isolated by identification of plaques expressing β-galactosidase. Disruption of Bm33 was confirmed by PCR (Fig. 3b). Successful isolation of BmORF33D showed that Bm33 is not essential for virus replication in BmN cells.
Next, we examined the effect of Bm33 deletion on BV production. BmN cells were infected with wt BmNPV (T3) or BmORF33D at an m.o.i. of 5, and yields of BV were determined by plaque assay. BmORF33D exhibited a similar rate of BV production in BmN cells compared with wt BmNPV (Fig. 4a), suggesting that Bm33 is not involved in BV production in BmN cells. Furthermore, we investigated the effect of Bm33 deletion on OB production in BmN cells, and observed that OB production was indistinguishable between wt BmNPV and BmORF33D (Fig. 4b). OB release into the culture medium occurred normally in BmORF33D-infected BmN cells, suggesting that Bm33 is not required for normal OB production in BmN cells.

Effect of Bm33 deletion on viral gene expression

We next examined the effect of Bm33 deletion on viral gene expression in BmNPV-infected BmN cells. Real-time PCR analysis showed that expression levels of the immediate early gene ie-1 (Fig. 5a), the late gene v-cath (Fig. 5b) and the very late gene polh (Fig. 5c) in BmORF33D-infected BmN cells were similar to those observed in wt BmNPV-infected cells. These results suggest that Bm33 has little effect on the levels of viral gene expression during BmNPV infection.

Generation and characterization of a Bm33-null BmNPV

As described above, BmORF33D had no obvious phenotype in BmN cells. However, a truncated form of Bm33 produced in BmORF33D-infected BmN cells might be functional because BmORF33D still encodes a helicase C-terminal domain of Bm33 (Figs 1a and 3a). Thus, we generated a Bm33-null BmNPV, BmORF33null, that lacked the entire Bm33 region (Fig. 3a), and examined BV and OB production in BmN cells at 48 h p.i. BV and OB production were indistinguishable between wt BmNPV
and BmORF33null (data not shown), suggesting that neither of the domains of Bm33 is required for normal production of BVs and OBs in BmN cells.

**Effect of Bm33 deletion on infectivity in B. mori larvae**

We next investigated whether Bm33 deletion had an effect on the infectivity of BmNPV in *B. mori* larvae. To determine the LD$_{50}$ of BV, fifth-instar larvae were injected haemocoelically with various doses of BV and monitored for mortality (Table 1). No significant differences among the LD$_{50}$ values were observed for wt BmNPV, BmORF33D and BmORF33null, suggesting that the infectivity of the BV form of Bm33-deletion mutants was normal in *B. mori* larvae. We next assessed the LT$_{50}$ by injecting BVs into fifth-instar larvae (1500 p.f.u. per larva). The LT$_{50}$ values of BmORF33D and BmORF33null were found to be approximately 10 h longer than that of wt virus (Table 2).

To determine the LC$_{50}$ of OBs, third-instar larvae were infected orally with selected concentrations of OBs and monitored for mortality (Table 1). No significant differences in the LC$_{50}$ values were observed among wt BmNPV, BmORF33D and BmORF33null, suggesting that the infectivity of the occluded form of BmORF33D and BmORF33null is normal in *B. mori* larvae. Third-instar larvae were then infected orally with a lethal concentration (1.3 × 10$^8$ OBs ml$^{-1}$) to see whether LT$_{50}$ was delayed in oral infection. The LT$_{50}$ values of BmORF33D and BmORF33null in oral infection were approximately 15 h longer than that of wt BmNPV (Table 2).

To verify that a mutation elsewhere in the genome was not involved in the observed phenotype of BmORF33D or BmORF33null, we examined the LT$_{50}$ of BmORF33His by injecting BVs into fifth-instar larvae (1500 p.f.u. per larva). We observed no significant difference between the LT$_{50}$ values of wt BmNPV and BmORF33His (data not shown). These results suggested that Bm33 is involved in viral pathogenicity in *B. mori* larva.

**DISCUSSION**

Comparative studies of baculovirus genomes have revealed that there are 12 genes exclusive to group I NPVs (Ikeda et al., 2006). Among them, five genes have been functionally characterized: *ie-2* encodes an ubiquitin ligase and is involved in cell-cycle arrest of host cells (Imai et al., 2003; Prikhod’ko & Miller, 1998); *gp64* encodes a major envelope glycoprotein of BVs that is required for host-cell receptor binding (Monsma et al., 1996); *ptp* induces abnormal behaviour of host insects at the late stage of infection (Kamita et al., 2005); BV/ODV-E26 is a structural protein of the envelope of both BV and ODV (Beniya et al., 1998), and co-localizes with IE-1 during infection (Kang et al., 2005); and *Bm21*, a homologue of **ac30**, is required for fast killing of *B. mori* larvae (Huang et al., 2008). In this study, we examined the role of one of these genes, Bm33, in BmN cells and *B. mori* larvae. Based on the similarity of Bm33 with the SNF2 family of proteins, this protein was suspected to play important roles in virus replication, presumably by opening up viral chromatin and aiding in binding of virus-specific transcription factors to viral DNA (Lapointe et al., 2000). However, we successfully isolated BmORF33D and did not observe any obvious defects in the production of BVs or OBs, suggesting that Bm33 is not essential for virus replication in cultured cells.
Larval bioassays showed that disruption of Bm33 did not reduce virus infectivity, but BmORF33D took 10–15 h longer to kill larvae than wt BmNPV, indicating that Bm33 is a viral pathogenicity factor in larvae infected with BmNPV. Several knockout NPVs with phenotypes similar to BmORF33D have been reported in BmNPV and AcMNPV. Bm21 is not essential for virus replication, but deletion of the gene delays killing of the infected larvae (Huang et al., 2008). Bm56 (ac68) is a baculovirus core gene and its deletion in BmNPV or AcMNPV does not affect viral infectivity but extends the LT50 value (Li et al., 2008; Xu et al., 2008). Ac23, an envelope fusion protein homologue encoded by group II NPVs, is not required for viral replication or pathogenesis in vitro or in vivo, but it accelerates death of the infected host (Lung et al., 2003). Although the functions of these genes within the host remain unclear, they could be categorized as viral virulence factors for host insects. Further knockout studies of NPV genes may identify additional viral virulence factors for insect larvae.

Although viral SNF homologues have not been discovered to date from any viruses other than group I NPVs, the SWI/SNF chromatin-remodelling complex is a co-factor for Tat transactivation of the human immunodeficiency virus promoter (Mahmoudi et al., 2006). In this study, we found that Bm33 acts as a viral virulence factor in insect larvae, but not in cultured cells. Bm33 may be required for activation of host and/or viral genes in B. mori larvae, which results in increasing the speed of killing of host larvae.

Transcriptional analysis showed that Bm33 was transcribed from 4 h p.i. and accumulated to high levels by 24 h p.i. (Fig. 2a). Lapointe et al. (2000) reported a transcriptional analysis of an SNF2 homologue of Choristoneura fumiferana NPV (CfMNPV). Northern blot analysis showed a strongly transcribed 2.1 kb transcript at 6 h p.i. and the level of this transcript increased in intensity up to 48 h p.i. in CfMNPV-infected Cf-124T cells. They also observed that some transcripts initiated upstream of lef-12 and overlapped the ORF of this gene. This temporal expression pattern is similar to that observed in BmNPV-infected cells (Fig. 2a), suggesting that Bm33 might be expressed as larger transcripts starting from a region upstream of lef-12 at the late stage of infection. 5'-RACE analysis revealed that the major start site of Bm33 mRNA was 42 nt upstream of the translation start codon, and mapped to CAAT but not to two CAGT consensus initiation motifs (Fig. 2b). Whilst the CfMNPV homologue promoter region contains one CAGT motif, the leader sequence of the AcMNPV homologue

Table 1. Dose–mortality of BmNPV T3, BmORF33D and BmORF33null in B. mori larvae

<table>
<thead>
<tr>
<th>B. mori/virus</th>
<th>LD50 (p.f.u.) or LC50 (OBs ml⁻¹)</th>
<th>95% Fiducial limit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>Fifth instar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BmNPV T3</td>
<td>9.1 x 10⁻²</td>
<td>4.8 x 10⁻²</td>
</tr>
<tr>
<td>BmORF33D</td>
<td>1.0 x 10⁻¹</td>
<td>6.3 x 10⁻²</td>
</tr>
<tr>
<td>BmORF33null</td>
<td>6.4 x 10⁻²</td>
<td>3.7 x 10⁻²</td>
</tr>
<tr>
<td>Third instar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BmNPV T3</td>
<td>1.1 x 10⁶</td>
<td>5.4 x 10⁶</td>
</tr>
<tr>
<td>BmORF33D</td>
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<td>1.4 x 10⁷</td>
</tr>
<tr>
<td>BmORF33null</td>
<td>2.6 x 10⁷</td>
<td>1.6 x 10⁷</td>
</tr>
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</table>

Table 2. Time–mortality of BmNPV T3, BmORF33D and BmORF33null in B. mori larvae

<table>
<thead>
<tr>
<th>B. mori/virus</th>
<th>Concentration</th>
<th>LT50 (h)</th>
<th>sd</th>
<th>95% Fiducial limit (h)</th>
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<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>Fifth instar</td>
<td>1500 p.f.u. per larva</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BmNPV T3</td>
<td>111.0</td>
<td>107.9</td>
<td>114.1</td>
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<tr>
<td>BmORF33D</td>
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<td>118.2</td>
<td>125.8</td>
<td></td>
</tr>
<tr>
<td>BmORF33null</td>
<td>120.2</td>
<td>117.5</td>
<td>122.9</td>
<td></td>
</tr>
<tr>
<td>Third instar</td>
<td>1.3 x 10⁸ OBs ml⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BmNPV T3</td>
<td>118.8</td>
<td>111.5</td>
<td>126.1</td>
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<tr>
<td>BmORF33D</td>
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<td>126.7</td>
<td>138.3</td>
<td></td>
</tr>
<tr>
<td>BmORF33null</td>
<td>133.0</td>
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</table>
carries no early or late start sites (Ayres et al., 1994; Lapointe et al., 2000). Reporter-based experiments are needed to examine whether viral factors, such as IE-1, are required for the transcription of SNF2 homologue genes, including Bm33.

In conclusion, we have reported for the first time functional characterization of the baculoviral SNF2 homologue Bm33. We found that it was not essential for virus growth in vitro or in vivo. Larval assays showed that it accelerated the death of B. mori larvae either by BV injection or by OB ingestion, suggesting that Bm33 plays an important role in systemic infection. To clarify the mechanism by which this protein accelerates mortality, we are currently analysing the effect of Bm33 deletion from the BmNPV genome on host and/or viral gene expression in infected B. mori larvae.

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