Characterization of a Late Gene, ORF134, from *Bombyx mori* Nucleopolyhedrovirus

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Key Words

*Bombyx mori* nucleopolyhedrovirus • *Bombyx mori* 134 • Transcription • Protein expression • Subcellular localization

Abstract

**Objective:** ORF134 of *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a homolog of *Autographa californica* multiple NPV ORF5, but its function is unknown so far. The aim of this study is to characterize BmNPV ORF134 (Bm134). **Methods:** Transcription, protein expression and subcellular localization of Bm134 in BmN cells and silkworm midguts were analyzed using real-time polymerase chain reaction (RT-PCR), Western blot and immunofluorescence, respectively. **Results:** Both the transcription and protein expression of Bm134 were not detected until 24 h post-infection (p.i.) by RT-PCR and Western blot, indicating that Bm134 is a late gene. Western blot revealed that Bm134 encodes an expected 12.4-kDa structural protein that is associated with occlusion-derived virus (ODV), not with budded virus. Immunofluorescence analysis showed that the Bm134 was first detected in the cytoplasm 24 h p.i. and then transported to the nucleus during later infection. Transcripts of Bm134 and the corresponding protein were only detected 48–72 h p.i. in BmNPV-infected larvae of 306, a highly BmNPV-susceptible silkworm strain, but not in the NB strain that is resistant to BmNPV infection. **Conclusion:** Taken together, our data suggest that Bm134 is a late gene of BmNPV and may function as an ODV structural protein.

Introduction

Baculoviridae are a large family of viruses that are pathogenic to invertebrates, particularly insects from the order of Lepidoptera, and thus are considered as a natural agent to control insect pests [1–3]. They are also exploited as protein expression and gene therapy vectors in insect cells and caterpillars [4, 5].

The baculovirus genome consists of a circular double-stranded DNA molecule of about 81.7–178.7 kbp, and they are usually subdivided into two genera: nucleopolyhedrovirus (NPV) and granulovirus (GV) [6]. Compared to GV, NPVs have been studied much more intensively due to their ability to be propagated in suitable cell lines. NPVs typically produce two kinds of progeny virus: occlusion-derived virus (ODV) and budded virus (BV). ODV transmits infection from insect to insect by infecting midgut columnar epithelial cells, whereas BV is responsible for causing systemic infection within the host [7]. They have different viral compositions despite containing an identical genome [8, 9]. ODV envelope proteins have been shown to be required to fuse with columnar cell surface proteins after occlusion bodies enter the midgut of susceptible insects [10].

The *Bombyx mori* NPV (BmNPV) is a major pathogen of the mulberry silk worm, and can cause a disastrous effect on sericulture [1]. It is also second only in popularity to *Autographa californica* nucleopolyhedrovirus (AcMNPV) as a baculovirus expression system. The
BmNPV (T3 strain) genome has been completely sequenced [11]. A number of BmNPV genes have been characterized, such as p95 [12], DBP [13], iel [14], BRO [15], Bm8 [16], Bm68 [17], Bm60 [1], Bm51 [18], Bm67 [19], Bm122 [20], and Bm79 [21]. Nevertheless, the functions of many other genes in the BmNPV genome still remain unknown, including orf134. In this study, we describe the transcription, expression and subcellular localization of the protein product of BmNPV ORF134 (Bm134) in BmN cells, a highly susceptible silkworm strain 306, and a resistant silkworm strain NB.

Materials and Methods

Viruses, Cells and Insects
BmNPV (T3 strain) virus was propagated in BmN cells which were maintained at 27° in TC-100 media supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL, Carlsbad, Calif., USA). The titration of virus and other routine manipulations were performed according to the standard protocol [22].

The silkworm B. mori was inbred in our laboratory. The highly BmNPV-susceptible silkworm strain 306 and the BmNPV-resistant silkworm strain NB were used for this study.

Computer-Assisted Sequence Analysis
The protein sequence was analyzed using software from ExPASy (www.expasy.org) for the prediction of motifs, domains, transmembrane regions and signal peptides [23]. Homologs were explored using the BLASTP searching tool in the updated GenBank/EMBL and SWISS-PROT databases [24, 25]. The sequence alignment was carried out with ClustalW (http://www.ebi.ac.uk/clustalw) and edited with Genedoc [26].

Transcription Analysis
First, BmN cells were infected with BmNPV at a MOI of 10. Total RNAs from transfected cells were isolated at mock, 0, 6, 12, 24, 36, 48, 72 h post-infection (p.i.) with Trizol reagent (Invitrogen, Carlsbad, Calif., USA) according to manufacturer’s protocol. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and quantified by optical density measurement at 260 nm. Total RNAs from transfected cells were isolated at mock, 0, 6, 12, 24, 36, 48, 72 and 96 h p.i. Protein samples of the midgut were analyzed by 15% SDS-PAGE and subsequently subjected to Western blot analysis.

Materials and Methods

Nucleotide and Amino Acid Sequence Analysis
The Bm134 coding region was amplified from the BmNPV genomic DNA by PCR using an upstream primer (5′-CGGGATCC ATGTATAGCACGTCAAAAATTAAC-3′) and a downstream primer (5′-CGGCTCGAGTTAATCGTGCGATCCTGCAAT-3′) with a BamHI site (underlined) and a HindIII site (underlined). The Bm134 was subcloned into the pET30a(+) expression vector (Novagen, USA) in frame with the N-terminal 6×His tag. The recombinant plasmid, pET-Bm134, was verified by PCR, restriction analysis and DNA sequencing. The recombinant plasmid was transformed into Escherichia coli BL21 (DE3) cells for protein expression under the induction conditions of 1 mM isopropyl-D-1-thiogalactopyranoside (IPTG) at 37° for 6 h. The 6× His-tagged recombinant Bm134 protein was purified on a Ni2+-NTA column (Novagen) and used to raise polyclonal antibodies in rabbits. The antibody was prepared using the standard technique [27], and was purified with protein A affinity column (Millipore).

Mass Spectrometry Analysis
The protein Bm134 was separated by 15% SDS-PAGE gel and then protein spots were manually excised from gels. Spots from Coomassie gels were washed with 100 ml of 50% acetonitrile/50 mM ammonium hydrocarbonate (pH 8) [28]. Gel pieces were then dehydrated with acetone and vacuum dried. After rehydration in 10 μl of 50 mM ammonium hydrocarbonate (pH 8), samples were incubated in the same buffer containing 0.5 μg of porcine trypsin (Promega, France) overnight (16–18 h) at 37°. Peptide fragments from digested proteins were then crystallized with α-cyano-4-hydroxycinnamic acid as a matrix and subjected to MALDI-TOF (Bruker Daltonics, Germany) for peptide mass fingerprinting. The peak lists were the basis for peptide mass fingerprint analysis by the Mascot software (Matrix Science; http://www.matrixscience.com/search_form_select.html).

Western Blot Analysis
BmN cells were infected with BmNPV T3 strain BVs (MOI 10) at the designated times (0, 6, 12, 24, 36, 48 and 72 h p.i.), and then were washed three times with cold phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4). The protein concentrations of the cell extracts were determined by the Bradford method [29]. Cell lysates (20 μg) were analyzed by 15% SDS-PAGE and subsequently subjected to Western blot analysis.

The 5th-instar larvae of 306 and NB were orally fed with 5 μl (TCD50/ml = 10³) of BmNPV and the midguts were harvested 12, 24, 36, 48, 72 and 96 h p.i. Protein samples of the midgut were separated on 15% SDS-PAGE and further analyzed by Western blot, as described previously [30].
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**Preparation of BV and ODV**

Fifth-instar larvae of 306 and NB were orally fed with 5 μl (TCD$_{50}$/ml = 10$^9$) of BmNPV T3 BVs. The infected larvae were fed at 25°C for 5 days, and the polyhedra were purified and verified by ordinary microscopy [31]. ODV was released from the occlusions by using alkaline treatment and purified from polyhedra as described by Braunagel and Summers [32] and Caballero et al. [33].

Hemolymph-derived BVs were purified from BmNPV-infected larvae as described previously [34] with minor modifications. Briefly, 3 days p.i. hemolymph was collected and clarified by centrifugation of 3,000 g for 5 min at 4°C. The supernatant was further filtered (0.45-μm filter, Millipore) and loaded onto a 25–56% (wt/wt) continuous sucrose cushion in 0.1×TE and centrifuged at 100,000 g for 90 min at 4°C. The BV band was collected and diluted in 0.1×TE, which was followed by centrifugation at 100,000 g for 90 min at 4°C and resuspended in 0.1×TE.

**Subcellular Localization Analysis of Bm134 Proteins in Infected Cells**

Monolayers of BmN cells infected with BmNPV (MOI 10) were collected at designated time points (24, 48 and 72 h). The cells were washed three times in PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. The cells were washed three times and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, and again washed three times with PBS. The cells were incubated with the anti-Bm134 antibody (dilution factor 1:5,000) for 1 h at room temperature. The anti-Bm134 antibody was removed by washing three times with PBS. Then cells were incubated with the secondary antibody, FITC-conjugated goat anti-rabbit IgG (Sigma, USA), and then DAPI (Sigma, USA) for 1 h at 37°C. Background staining was removed by washing with PBS three times. The cells were examined and photographed under a Zeiss LSM 5 Live (Carl Zeiss, Germany) confocal laser scanning microscope for fluorescence.

**Results**

**Sequence Analysis of Bm134**

The Bm134 contains 330 nucleotides and encodes a 109 amino acid peptide with a predicted molecular mass of 12.4 kDa (http://www.expasy.ch/tools/pi_tool.html). A putative late transcription motif, GTAAG, was found at 43 nts upstream of the start codon, ATG, suggesting that Bm134 might be a late transcriptional gene. PROSITE was used to predict the translational modifications and functional motifs of the Bm134 protein. Five protein kinase C phosphorylation sites (aa 4–6, aa 30–32, aa 65–67, aa 83–85 and aa 87–89) and two casein kinase II phosphorylation sites (aa 15–18, aa 44–47) were found in the Bm134 amino acid sequence. Signal peptide sequence, transmembrane region, nuclear localization signal, or membrane retention signal could be detected by ExPASy tools.

Burst search showed that Bm134 homologs exist in 13 baculovirus NPVs with identities ranging from 93 to 31%. Sequence comparison showed that Bm134 has the highest identity (93%) with AcMNPV orf5 and the lowest identity (31%) with IrfaNPV Ac5-like protein (fig. 1). Only one homolog was found in GV.

**Transcriptional Analysis of Bm134**

To analyze the Bm134 transcription, RT-PCR was performed using total RNA isolated from BmNPV-infected BmN cells as template. We used the BmNPV ie1 gene and p10 gene as controls for the early gene and very late gene, respectively. As expected, a band with an expected size of 180 bp for Bm134 could be detected by PCR 24 h p.i., which remained detectable up to 72 h p.i. Similarly, a 193-bp p10 fragment was detectable from 24 to 96 h p.i. By contrast, a 315-bp ie1 fragment was detectable from 6 to 72 h p.i. (fig. 2). We also performed qRT-PCR analysis showing that the transcript of Bm134 was detected from 24 to 72 h p.i., and reached maximal levels 72 h p.i. (fig. 3a).

The transcription levels of Bm134 in BmNPV-infected larvae of 306 and NB were also examined by qRT-PCR. Interestingly, transcripts of Bm134 were detected 48–72 h p.i. in BmNPV-infected larvae of 306, but not in NB (fig. 3b). Together, our data suggest that Bm134 is a late gene.

**Expression of Bm134, Mass Spectrometry Analysis and Western Blot**

To prepare antibody against Bm134, the 6× His-tagged recombinant Bm134 protein was expressed in *E. coli*. Western blot analysis using anti-His antiserum confirmed that the 19-kDa Bm134 was successfully expressed (fig. 4). Mass spectrometry further confirmed that the 19-kDa protein was the 6× His-Bm134 (fig. 5).

To further confirm that Bm134 is a late gene, we did Western blot to detect the protein expression in BmNPV-infected BmN cells, 306 and NB. A specific immunoreactive band of approximately 12.4 kDa, which is in agreement with Bm134 predicted molecular weight, was first observed 24 h p.i. and remained detectable up to 72 h p.i. in BmNPV-infected BmN cells (fig. 6a). In addition, an immunoreactive band in the midguts of BmNPV-infected 306 was first observed 48 h p.i., but not observed in NB (fig. 6b). These data are consistent with the analysis of transcripts and indicate that Bm134 was synthesized at a late stage of infection.
Immunodetection of the Bm134 Protein in BVs and ODVs

To investigate whether the Bm134 protein was a virus structural protein, Western blot analysis of purified BVs, ODVs and BmNPV-infected BmN cells (as control) were carried out (fig. 7). The BmNPV-infected cells and ODV fraction showed a reactive band. In contrast, no band was observed in the BV samples, indicating that the product of Bm134 encodes a structural protein associated with the ODV. The preparation of infected cell, BV and ODV samples were examined by immunoassay with the anti-Bm51 antiserum.

Subcellular Localization of Bm134 Protein in Infected BmN Cells

To visualize the distribution of Bm134 during BmNPV infection, the subcellular localization of Bm134 protein was investigated by immunofluorescence using anti-Bm134 antibody. Since the Bm134 protein was first
detected 24 h p.i., we chose time points of 24, 48 and 72 h p.i. for observation. The result suggested that Bm134 was localized primarily in the cytoplasm and hardly present in the nucleus 24 h p.i., and was detected in the nucleus from 48 until 72 h p.i. (fig. 8). In the control, no obvious fluorescence signal was observed in virus-infected cells exposed to pre-immune serum and FITC-conjugated goat anti-rabbit IgG (fig. 8).

**Discussion**

In this study, we reported the characterization of a novel BmNPV gene Bm134, a gene that has thus far not been characterized. The search for homologs of Bm134 revealed that homologous genes were present in 12 members of lepidopteran NPVs, yet could not be detected from other members, suggesting that Bm134 and its homologs are specific to these NPVs.

The Bm134 gene contains the baculovirus consensus late start site motif GTAAG, indicating it could be a late gene. To clarify this, we performed RT-PCR and Western blot analysis. RT-PCR showed that Bm134 transcription started 24 h p.i. and accumulated until at least 72 h p.i. in BmNPV-infected BmN cells. Western blots of totally infected cell proteins also detected a single protein migrating at 13 kDa from 24 to 72 h p.i. by Bm134 polyclonal antibody. Therefore, our data suggest that Bm134 is indeed a late gene. It is known that Baculovirus gene ex-
Fig. 5. MALDI spectra of tryptic digest of recombinant Bm134. The identified protein, score, amino acid sequence coverage and the number of identified peptides are shown.

Fig. 6. Western blot analysis of the Bm134 protein in BmN cells (a) and 306 (b). The cells were collected 0, 6, 12, 24, 36, 48, 72 h p.i. The midguts of BmNPV-infected 306 were collected 12, 24, 36, 48, 72, 96 h p.i. and then processed for Western blot using anti-Bm134 antibody followed by incubation with a goat anti-rabbit IgG conjugated to HRP. The signal was detected with DAB substrate. Molecular mass standards are shown on the left.
**Fig. 7.** Immunodetection of Bm134 protein in BmNPV BVs, ODVs and BmN cells. BV, ODV, cell lysate samples were separated by SDS-PAGE and analyzed by Western blot. Lane 1 = Total proteins of infected cells; lane 2 = purified BV; lane 3 = purified ODV. The preparation of infected cells, BV and ODV samples were examined by immunoassay with the anti-Bm51 antiserum as control.

**Fig. 8.** Subcellular localization of Bm134 in BmNPV-infected BmN cells. The cells were collected 24, 48 and 72 h p.i., washed with 1× PBS and reacted with anti-Bm134 antibody followed by incubation with FITC-conjugated goat anti-rabbit IgG. Pre-immune serum was used as control. The nuclei were stained with DAPI (blue). Cells were photographed under a confocal laser fluorescence microscope.
pression can be divided into at least three phases [35]: early, late, and very late. The products of early genes are often involved in DNA replication, late gene expression, and host-modification processes [36]. Late proteins are frequently involved in the regulation of late and very late gene expression [37, 38], and most viral structural proteins were found to be late proteins [39]. Very late proteins are often involved in the processes of occlusion and cell lysis [40].

Transcriptional analysis and Western blots of Bm134 in the BmNPV-infected highly susceptible silkworm strain 306 further confirmed that Bm134 is a late gene, but transcripts of Bm134 in the BmNPV-infected resistant silkworm strain NB were not detected, suggesting that the expression of Bm134 in the BmNPV-infected NB silkworm was inhibited. Transcription and expression of Bm134 in BmNPV-infected 306 could be detected 48 h p.i., which was much later compared with that of BmNPV-infected BmN cells, indicating a delay of transcription of Bm134 in B. mori.

Immunodetection of Bm134 protein in BmNPV BVs, ODVs and BmN cells showed that an immunoreactive band was present only in ODV, but not in BV, which indicated that Bm134 is a specific ODV protein. ODV contains proteins essential for viral DNA replication, and many genes encoding ODV-associated proteins are conserved [41]. These results also imply that Bm134 may be involved in producing the nuclear viral envelopes to form ODV in the process of ODV formation.

The subcellular localization analysis revealed that Bm134 localized primarily in the cytoplasm 24 h.p.i. and both in the cytoplasm and nucleus 48 and 72 h.p.i. A similar protein localization pattern was found for other baculovirus proteins, such as Ha83 [42] and Bm60 [1], which may suggest a similar but unknown functional pattern.

In this study, we characterized an unknown BmNPV gene, Bm134. Our data provide competent evidence that Bm134 is a late gene and functions as ODV structural protein. It could be detected in BmNPV-infected B. mori cells or the midguts, but not in the BmNPV-resistant NB silkworm strain. Our work provides an excellent platform for subsequent in-depth analysis of its function and roles involved in BmNPV infection and anti-BmNPV response.

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