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Bm91 is an envelope component of ODV but is dispensable for the propagation of *Bombyx mori* nucleopolyhedrovirus

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ABSTRACT

Orf91 (*Bm91*) of *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a highly conserved gene that encodes a predicted 105-amino-acid protein, but its function remains unknown. In the current study, 5′-RACE revealed that the transcription initiation site of *Bm91* was – 12 nucleotides upstream of the start codon ATG, transcription of *Bm91* was detected from 12 to 96 h postinfection (p.i.) and Bm91 protein was detected from 24 to 96 h p.i. in BmNPV-infected BmN cells. Furthermore, Western blot analysis revealed that Bm91 was in occlusion-derived virus (ODV) but not in budded virus (BV). To investigate the role of *Bm91* in baculovirus life cycle, a *Bm91*-knockout virus was constructed by bacmid recombination in *E. coli*. Fluorescence and light microscopy showed that the production of BV and occlusion bodies (OBs) in *Bm91*-deficient-virus-infected BmN cells were similar to those in wild-type-virus-infected ones. Bioassay results showed that genetic deletion of *Bm91* did not significantly affect BmNPV infectivity, but extended the median lethal time (LT_{50}). Taken together, these results indicate that *Bm91* is not essential for viral propagation in *vitro*, but absence of the gene may affect the virulence of ODVs in silkworm larvae.

1. Introduction

Baculoviruses are enveloped, double-stranded DNA viruses that exclusively infect insects. The *Baculoviridae* are currently divided into four genera: *Alphabaculovirus* (lepidopteran-specific nucleopolyhedrovirus NPV), *Betabaculovirus* (lepidopteran-specific granuloviruses), *Gammabaculovirus* (hymenopteran-specific NPV), and *Deltabaculovirus* (dipteran-specific NPV) (Jehle et al., 2006). To date, the genomes of 57 baculoviruses have been sequenced and the genomes range in size from 80 to 180 kbp (Thiem, 2009; Miele et al., 2011). During the life cycle of Baculoviruses, two typical progeny virion forms are produced, budded virus (BV) and occlusion-derived virus (ODV). The two phenotypes are genetically identical, but differ structurally and functionally. BVs are responsible for spreading infections among cultured cells and tissues in the host, while ODVs are occluded in polyhedra or occlusion bodies and are required for the oral infection (Granados and Lawler, 1981; Keddie et al., 1989).

BmNPV is a representative member of *Alphabaculovirus*, which is a major pathogen of silkworm and causes serious economic damage to silk production. Since the genome of BmNPV was sequenced (Gomi et al., 1999) and published on NCBI, referred to (accession number: NC_001962), the research on BmNPV molecular biology has made explosive advances for many years, particularly the functions of specific genes. The genome of BmNPV is 128 kbp in length and contains 143 putative open reading frames (ORFs). Up to now, the functions of about 65% genes in BmNPV genome have been described, and the genes are classified into essential genes and auxiliary genes according to their functions (O'Reilly, 1997). In other words, essential genes are required for viral propagation such as *lef1*, *lef3*, *lef4*, *dnapol* (Mikhailov and Rohrmann, 2002; Hang et al., 1995; Knebel-Mörsdorf et al., 2006; Vanarsdall et al., 2005), while auxiliary genes can regulate the transcription of viral genes *in vivo* such as *Ac18*, *Ac34*, *Bm60* (Wang et al., 2007; Cai et al., 2012; Guo et al., 2010).

Bm91 locates at 87,452–87,769 nt in BmNPV genome and encodes a putative protein of 105 amino acids with a predicted molecular weight of 11.8 kD. A typical late promoter motif TAAG is −13 nt upstream of ATG that might initiate the transcription during the late phase of viral life. In addition, bioinformatical analysis showed that *Bm91* is a representative member of 11 k gene family (InterPro accession number: IPR009313) in insect viruses, and its homologs were found in a number of baculovirus genomes. These baculovirus proteins are all about 11 kDa in size, but their functions are unknown yet.

In this study, we examined the transcription and expression of *Bm91* in virus-infected BmN cells. We also investigated the structural localization and the role of Bm91 in viral life cycle. Our results indicate that *Bm91* is a late gene encoding an envelope component

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of ODV particle, and it is not an essential gene for BmNPV replication in vitro.

2. Materials and methods

2.1. Bacmid, bacterial strains, virus and cells

Bacmid containing a BmNPV genome (BmNPV bacmid) was propagated in *E. coli* strain DH10B, which was donated by Dr. EY Park (Department of Applied Biological Chemistry, Shizuoka University, Japan). *Escherichia coli* strains DH5α respectively containing plasmid pBADgbaA and pKOV-Cm, and the anti-AcMNPV VP39 rabbit polyclonal antibody, were kindly donated by Dr. Kai Yang (State Key Laboratory of Biocontrol, Sun Yat-sen University). *E. coli* Rosetta 2 (DE3) pLysS strains was maintained in our laboratory.

BVs were purified from the supernatant of BmNPV-infected BmN cells as described previously (Braunagel and Summers, 1994). Polyhedra were isolated from fifth-instar larvae of strain 306 (a highly susceptible silkworm to BmNPV) injected with BmNPV. ODVs were released from the occlusion bodies by using alkaline treatment and purified as described previously (Braunagel and Summers, 1994). BmN cells were grown at 27 °C in TC-100 insect medium supplemented with 10% fetal calf serum (Gibco).

2.2. Expression of Bm91 in E. coli and preparation of Bm91-specfic antibody

Two primers 91-F:5′-ATGGATCCATGAAACCGACGGC-3′ (*Bam*HI site was underlined) and 91-R:5′-GCAAGCTTTGTTGTTGCATTTCTATT-3′ (*Hin*dIII site was underlined) were designed to amplify the ORF of *Bm91* from the BmNPV genomic DNA. The PCR product was cloned into the expression vector pET30a (Novagen, USA) resulting in pET30a-*Bm91* with 6 × His-tag at the N terminus, which was confirmed to be correct by restriction analysis and DNA sequencing. Subsequently, the recombinant plasmid pET30a-*Bm91* was introduced into host strains rosettaTM 2 (DE3) pLysS cells and was cultured in LB medium in 37 °C. When the culture reached an OD600 of 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) at the concentrations ranging from 0.2 to 0.8 mmol/L was added to induce the expression of Bm91 protein. After the cultures were grown for 10 h, then cells were harvested from medium by centrifugation at 7000*g* for 10 min at 4 °C. The fusion protein present in the cell pellet was separated in 15% SDS polyacrylamide gels and stained with Coomassie brilliant blue. The induced Bm91 band was excised directly and antiserum were raised in rats according to the method of Sambrook using Freund's adjuvant (Sambrook, 2001).

2.3. Transcription analysis of Bm91

1.6×10^6 BmN cells plated in 6-well plate were infected with BmNPV at a multiplicity of infection (MOI) of 5 plaque forming units (PFU) per cell, and cells were respectively collected at 0, 3, 6, 12, 24, 48, 72 and 96 h p.i. Total RNA was extracted using the TRIZOL® Reagent from Invitrogen according to the manufacturer's instructions. The RNA samples were quantified by optical density measurements at 260 nm.

After treatment with RNase-free DNaseI, RT-PCR was carried out using ThermoScript™ RT-PCR system Kit (Invitrogen) with 3 μg treated mRNA as template. First-strand cDNA synthesis was performed using an avian reverse transcriptase and oligo$(dT)_{20}$ according to the manufacturer's instructions. The cDNA product was amplified by the gene-specific primers 91-F and 91-R. BmNPV *ie1* gene and *vp39* gene were used as the control for early gene and late gene, respectively. The primer pair *ie1*-F: 5′-AACGCGTCGTACACCAGT-3′ and *ie1*-R: 5′-CCGTGCAAATGTTCGTGT-3′ were designed for *ie1*; The primer pair: *vp39*-F: 5′-GCCGCGACAAA TGAGAGT-3′ and *vp39*-R:5′-GTTCGGGTTTGTGGTGTC-3′ were designed for *vp39* gene. Negative control experiments were performed to detect any possible viral DNA contamination using H_2O and treated mRNA as template respectively. *Bm91* RT-PCR products were gel purified and cloned into the pMD18-T vector (TaKaRa) for sequencing.

The 5′ end of *Bm91* transcript was determined with the 5′ rapid amplification cDNA ends (RLM-RACE) kit (Ambion) according to the Ambion instructions. The first PCR was performed with 5′ RACE outer primer: 5′-GCTGATGGCGATGAATGAACACT G-3′ and GSP1: 5′-GCAAGCTTTGTTGTTGCATTTCTATT-3′, and nested PCR was carried out to amplify the 5′ end of Bm91 transcript with 5′ RACE inner primer: 5′-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3′ and GSP2: 5′-TGTTTTTGTACAGTAACTGTTTCTTGGTT-3′. The PCR products were gel purified and cloned into pMD18-T (TaKaRa), then sequenced with M13F(-47) or M13R(-48) primers.

2.4. Western blot analysis

For expression phase analysis of Bm91 protein, a monolayer culture of BmN cells (1.6×10^6) plated in 6-well plate were infected with mock or BmNPV (m.o.i. of 5). Cells were harvested at 0, 3, 6, 12, 24, 48, 72, and 96 h p.i. respectively, then the cell pelletes were resuspended in 100 μl of phosphate-buffered saline (PBS, pH 7.4) and boiled for 10 min. Lysates were clarified by centrifugation at 15,000*g* for 10 min. To address whether Bm91 is a viral structural protein or not, total protein from purified ODVs and BVs were subjected to Western blot analysis respectively. Additionally, Envelope (E) and nucleocapsid (NC) were further separated from purified ODV according to the method of Braunagel and Summers (1994). Briefly, Purified ODVs were suspended in 1.5% Nonidet P40 (NP-40), TBS buffer and incubated in ice for 30 min. Suspension was centrifugated at 35,000 rpm for 45 min at 4 °C. The NC proteins were harvested after the harvested pellet was washed with 0.1 × TE and centrifugated at 35,000 rpm for 45 min at 4 °C. The E proteins were harvested after the supernatant were acetone-precipitated and concentrated by centrifugation (35,000 rpm, 45 min) twice, and the pellet was dissolved in 0.1 × TE.

The protein concentrations from cell lysates and purified virion extracts were determined by Bradford's method (Bradford, 1976). 30 μg of protein were subjected to SDS–PAGE and transferred onto polyvinyldifluorene (PVDF) membrane (Millipore cat. No. IPVH00010). The blots were blocked with 5% nonfat milk in 1 × PBST for one hour and then incubated with Bm91 antibodies at a dilution of 1:1000 in 5% nonfat milk in PBST. They were then washed with PBST three times and subsequently, the membrane was incubated with goat anti-rat IgG conjugated to horseradish peroxidase diluted 1:5000 for 1 h at room temperature. The amino acid identity is 95% between BmNPV VP39 and AcMNPV VP39, so the anti-AcMNPV VP39 rabbit polyclonal antibody could be exploited to detect the BmNPV VP39, which was used as a positive control to indicate the quality of extracts from BV and ODV. The hybridization signal was visualized by using diaminobenzidine (DAB) substrate solution.

2.5. Generation of the Bm91-knockout BmNPV bacmid

A *Bm91* knockout BmNPV bacmid was generated in *E. coli* by ET recombination system as described previously (Li et al., 2008). A transfer vector in which the *Bm91* locus region was replaced with a chloramphenicol resistance (Cm) gene for antibiotic selection in *E. coli*, was constructed as follows. Two primers Cm-F: 5′-GGATCCCTTCGAATAAATACCTGTGA-3′ (*Bam*HI was underlined)