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Characterization of ORF29 of Bombyx mori nucleopolyhedrovirus

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Summary. – ORF 29 of Bombyx mori nucleopolyhedrovirus (BmNPV ORF29), a conserved gene among all the lepidopteran baculoviruses, was subjected to analysis of sequence and transcription/translation in the insect BmN cells *in vitro*. His-tagged BmNPV ORF29 fusion protein was expressed in *Escherichia coli,* purified and used for the production of an antiserum. All the homologs of BmNPV ORF29 in baculoviruses contain a Nudix motif with slight modifications in the N-terminal part. A 654 nts-long transcript of BmNPV ORF29 in BmN cells was detected at 3–48 hrs p.i. indicating the early nature of the gene. In the virus-infected cells, the BmNPV ORF29 protein of 26 K was present in both the cytoplasm and nucleus. In the virions, the protein was present in Budded virus (BV), but not in Occlusion-derived virus (ODV). These results suggest that BmNPV ORF29 is a functional ORF of BmNPV, which encodes a 26 K protein expressed in the early stage of infection cycle.

Keywords: Bombyx mori nucleopolyhedrovirus; ORF29; gene; transcript; protein

Introduction

The family *Baculoviridae* comprises arthropod-specific insect viruses with a large, circular, supercoiled, double-stranded DNA genome present in occluded rod-shaped virions (Moscardi, 1999). Baculovirus infection cycle is characteristic of the early, late, and very late phases for DNA replication, gene transcription, and protein synthesis. Two types of virions are produced during the life cycle, ODV and BV. The ODV transmits infection from insect to insect by infecting midgut columnar epithelial cells, whereas the BV is responsible for systemic infection within the host (Keddie *et al*., 1989).

Since the first baculovirus was completely sequenced, 29 other baculovirus genomes have been reported so far (Ayres *et al*., 1994). BmNPV contains a covalently closed circular genome of 128 kbp containing about 143 putative ORFs based on the criterion that an ORF should be a single, contiguous, non-overlapping coding region (Jehle *et al*., 2006). BmNPV infects the mulberry silkworm larvae, what presents a major economic problem in tropical sericulture. Among these ORFs, BmNPV ORF29 was found to belong to a unique Nudix hydrolases superfamily that are widely distributed in natural world and have been identified in all species from viruses to humans (McLennan, 2006). These enzymes are characterized by the presence of a highly conserved sequence motif called the "Nudix motif" $GX_5EX_7REUXEEXGU$, where U is a bulky hydrophobic aa usually Ile, Leu, or Val, and X can be any residue (Bessman *et al*., 1996).

Proteins with Nudix motif have been identified in some other viruses including vaccinia D9 and D10 Nudix genes, African swine fever virus g5R (also known as D250) with little knowledge about their function in the virus replication. Earlier studies demonstrated that D9 is not essential for the viral replication in cultivated cells (Dvoracek and Shors, 2003). The vaccinia D10 may have a role in a cap structure metabolism and affects the stability or translatability of mRNAs (Parrish and Moss, 2006). g5R was suggested to regulate a stage of viral morphogenesis involving diphosphoinositol polyphosphate-mediated membrane trafficking (Cartwright *et al*., 2002).

BmNPV ORF29 is located between nts 26,451 and 27,102 in the BmNPV genome and encodes protein of 217 aa residues with predicted Mr of 26 K. It is transcribed in the opposite orientation of polyhedrin gene. BmNPV ORF29

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Abbreviations: ADPR = ADP-ribose; BmNPV = Bombyx mori nucleopolyhedrovirus; BV = Budded virus; NPV = nucleopolyhedrovirus; ODV = Occlusion-derived virus; p.i. = post infection

is conserved in most baculoviruses infecting lepidopteran insects suggesting that it maybe involved in some basic processes of the viral life cycle (Jehle *et al.*, 2006). However, characterization of BmNPV ORF29 homologies has been reported in other viruses, but they have never been characterized in BmNPV. A BmNPV-based baculovirus expression system is a power tool for rapid and high-efficient expression of foreign genes, since the silkworm larvae that are easy to rear at low cost, can be used instead of the cultured cells (Sehgal and Gopinathan, 1998).

In this report, we tried to characterize BmNPV ORF29 by the transcriptional analysis, protein identification, and subcellular localization.

Materials and Methods

Virus and cell lines. Bombyx mori cell line (BmN) was maintained at 27°C in TC-100 insect medium supplemented with 10% fetal bovine serum (Gibco-BRL). BmNPV (T3 strain) was used for the infection as wild type and was propagated in BmN cells.

Computer-assisted sequence analysis. BmNPV ORF29 was analyzed using ExPASy server (www.expasy.ch) for the prediction of motifs, domains, transmembrane regions, and signal peptides (Appel *et al.*, 1994). Homologues were explored by using BLAST searching tool in the updated GenBank/EMBL and SWISS-PROT databases (Pearson, 1990; Altschul *et al.*, 1997). The sequence alignment was carried out with ClustalW (http://www.ebi.ac.uk/clustalw) and edited with Genedoc software Ver.2.04 (Free Software Foundation).

RT-PCR.. For the transcriptional analysis, BmN cells were infected with BmNPV at MOI = 10. Total RNA was isolated from the mock-infected and infected cells at 0, 3, 6, 9, 12, 24, and 48 hrs p.i. with Trizol reagent (Invitrogen). RNA was dissolved in DEPC-treated water and quantified by optical density measurement at 260 nm.

For cDNA synthesis and PCR experiment, the total RNA was first treated with DNase to eliminate any potential genomic DNA contamination. Total RNA (2 μg) from each time point p.i. was transcribed by AMV reverse transcriptase and oligo (dT) primer (Takara) to synthesize the first strand of cDNA. PCR was then carried out using BmNPV ORF29-specific primers of 5'-ATGCGAAACGCTGCA-3' and 5'-TTACACCGCCTAAG-3'.

His-tagged BmNPV ORF29 fusion protein and preparation of the specific antibody. BmNPV ORF29 coding sequence was amplified with primers 5´-AGGATCCATGCGAAACGCTGCA-3' (containing the *Bam*HI site) and 5'-GCTCGAGTTACACCCGCCTAAG-3' (containing the *Xho*I site) from the BmNPV genomic DNA by PCR. The BmNPV ORF29 was subcloned into the pET30a (+) expression vector (Novagen) in frame with the N-terminal 6×His tag. The recombinant plasmid pET-BmNPV ORF29 was verified by PCR, restriction analysis, and DNA sequencing. Then, it was transfected into *Escherichia coli* BL21 cells for the expression. The 6×His-tagged recombinant BmNPV ORF29 protein was purified on a Ni^{2+}-NTA column (Novagen).

The New Zealand white rabbits were subcutaneously injected with the purified protein mixed with complete Freund's adjuvant (Sigma) followed by two booster injections of protein mixed with incomplete Freund's adjuvant (Sigma) within a gap of 2 weeks before exsanguination. The obtained polyclonal rabbit antibody against 6×His-BmNPV ORF29 was used in the immunoassay.

In gel digestion and peptide analysis by MALDI-TOF. The protein bands in Coomassie Blue stained gel were digested with trypsin and analyzed by matrix-assisted laser desorption ionization-time-of flightmass spectrometry (MALDI-TOF) (Bruker Daltonics) that generated a peptide sequence in addition to the peptide mass information. The mass values of tryptic peptides obtained by MALDI-TOF were used as inputs to search the corresponding proteins in database NCBInr and MSDB via the program Mascot.

Western blot analysis. For the time course analysis, BmN cells were infected with BmNPV at MOI = 10. The infected cells were collected at designated times (mock, 0, 3, 6, 12, 24, 48 hrs p.i.) washed three times with PBS and lyzed in SDS-PAGE loading buffer by boiling for 10 mins. Protein samples were separated by SDS-PAGE and transferred onto a PVDF membrane in cold Towbin buffer (0.025 mol/l Tris, 0.19 mol/l glycine, 20% methanol). The membranes were blocked in 3% skimmed milk powder in PBST for 1 hr followed by the incubation with anti-BmNPV ORF29 polyclonal antiserum diluted 1:5,000 for 1 hr at room temperature. After washing the membrane was incubated with HRP-conjugated goat anti-rabbit IgG diluted 1:5,000 for 1 hr at room temperature. The signal was detected with DAB substrate solution (Chen *et al.*, 2007).

Purification of BV and ODV. Virions produced in the infected larvae of *Bombyx mori* were analyzed by electron microscopy and purified as described by Summers and Smith (Summers and Smith, 1978). The ODV was purified by ultracentrifugation on the sucrose gradient (Braunagel and Summers, 1994).

Hemolymph-derived BV was purified from BmNPV-infected larvae as described previously with some modifications (Palhan and Gopinathan, 1996). Three days p.i. the hemolymph was collected and clarified by centrifugation at 2,000 x g for 10 mins at 4°C. The supernatant was passed through a 0.45 μm pore filter. BV in the filtrate was centrifuged through a 25–56% (wt/wt) continuous sucrose gradient made up in 0.1x TE at 100,000 x g for 60 mins at 4°C and the pellet was resuspended in 0.1x TE. After purification the BV was incubated with 1% Nonidet-P40 and the fractions containing envelope material and nucleocapsids were separated by the centrifugation (Guarino *et al.*, 1995).

Immunofluorescence assay. Monolayers of BmN cells infected with BmNPV (MOI = 10) at 36 hrs p.i. were washed with cold PBS and fixed with 4% paraformaldehyde for 20 mins. The cells were then washed 3 times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 mins. After washing with cold PBS, the cells were incubated with BmNPV ORF29 polyclonal antiserum (diluted 1:50 in 3% BSA) for 2 hrs at 37°C. Cells were washed 3 times with PBS and then incubated with the secondary antibody, FITC-conjugated goat anti-rabbit IgG (Sigma) for 1 hr at 37°C. After three washes with PBS, diluted DAPI (Beyotime Biotechnology) staining solution was added to the coverslip preparations. Background staining was removed by triple washing with PBS. The cells were examined under a Leica confocal laser scanning microscope.

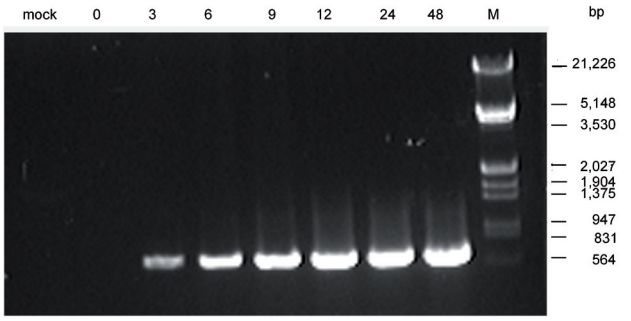

Fig. 1

Transcription of BmNPV ORF29 in BmN cells

RT-PCR analysis of the BmNPV ORF29 transcription in mock- and BmNPV-infected cells at 0, 3, 6, 9, 12, 24, and 48 hrs p.i. as indicated above the lanes. The bp values are shown on the right.

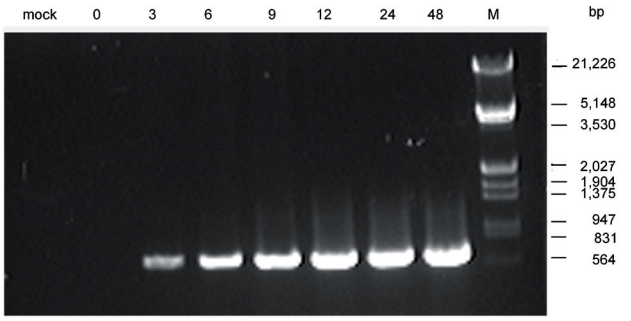

Fig. 2

Expression of His-tagged BmNPV ORF29 fusion protein in *E. coli*

SDS-PAGE (a) and Western blot analysis (b). Negative controls (lanes 1 and 2), the cells transfected with pET30a-BmNPV ORF29 (lanes 3 and 4), protein size markers (lane M). The arrow indicates fusion protein.

Results

Sequence analysis of BmNPV ORF29

The coding region of BmNPV ORF29 is 654 bp in length and could encode a 217-aa peptide with a predicted Mr of 26 K. Searches of protein databases GenBank and SWISS-PROT revealed that BmNPV ORF29 is conserved among all lepidopteran nucleopolyhedroviruses and granuloviruses, whose complete sequences have been reported, but not in baculoviruses Neodiprion abietis NPV, Neodiprion lecontii NPV, Neodiprion sertifer NPV, and Culex nigripalpus NPV. BmNPV ORF29 shared an identity of 96% with Plutella xylostella multiple nucleopolyhedrovirus ORF38 and Autographa californica multicapsid nucleopolyhedrovirus ORF38.

Querying BmNPV ORF29 sequence in the NCBI Conserved Domain Database showed a potential Nudix motif in the N-terminal part (Marchler-Bauer *et al.*, 2005). Multiple alignments revealed a conserved domain $GX_5EX_7REUXEEX_2U$ aligned with the confirmed conserved domain positions within other Nudix hydrolases.

Transcriptional analysis of BmNPV ORF29

To determine the time of BmNPV ORF29 transcription, RT-PCR analysis was performed using total RNA isolated from the BmNPV-infected host cells as a template. A band with the expected size of 654 bp was amplified from BmNPV-infected cells at 3 hrs p.i. and remained detectable up to 48 hrs p.i. (Fig. 1).

Expression of His-tagged BmNPV ORF29 fusion protein in E. coli

Expression of a 6×His–BmNPV ORF29 fusion in *E. coli* resulted in the production of a 32 K protein (Fig. 2). Western blot analysis using anti-His–BmNPV ORF29 antiserum confirmed that the 32 K protein was the fusion protein.

To further determine if the purified protein obtained after SDS-PAGE was BmNPV ORF29, the 32 K protein band was digested with trypsin and analyzed by MALDI-TOF mass spectrometry. The Mascot search was performed with carbamidomethyl as the fixed modification of cysteine and variable N-terminal Gln-pyroGlu. The 32 K protein was confirmed to be BmNPV ORF29 (Mascot score 184, 13 peptides matching, and 68% aa coverage). Then, the purified fusion protein was used to immunize rabbits to produce a specific antiserum against BmNPV ORF29.

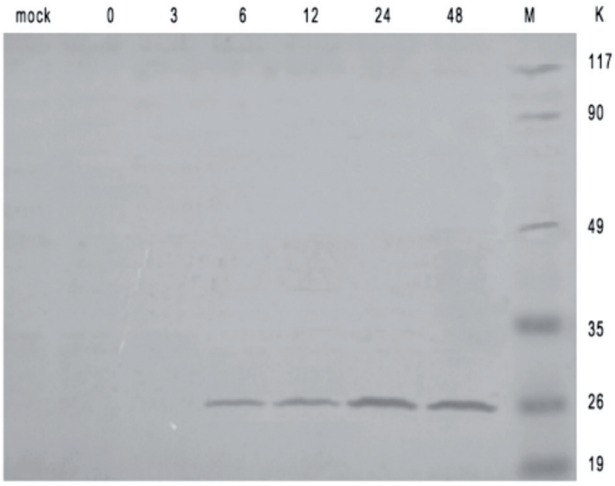

Fig. 3

Transcription of BmNPV ORF29 in BmN cells

Western blot analysis of the mock- and BmNPV-infected cells at 0–48 hrs p.i. using BmNPV ORF29 antiserum. Protein size markers are shown on the right.

Temporal expression of BmNPV ORF29 in the infected cells

In order to study the expression of BmNPV ORF29, a time course of BmNPV-infected cells were analyzed by the Western blot using anti-BmNPV ORF29 antiserum. A specific immunoreactive band of approximately 26 K was first observed in the BmNPV-infected cells at 6 hrs p.i. and remained detectable up to 48 hrs p.i. (Fig. 3). These data were consistent with the analysis of transcripts and indicated that BmNPV ORF29 was synthesized at an early stage of infection. No immunoreactive band was detected in the mock-infected cells. The size of immunoreactive band 26 K was in accord with the predicted Mr.

Localization of BmNPV ORF29 protein in virions

To investigate whether the BmNPV ORF29 protein was a structural protein, Western blot analysis of purified BV and ODV were carried out (Fig. 4). A clear band of 26 K was detected in the sample of purified BV. In contrast, no matching band was observed in the ODV samples indicating that the product of BmNPV ORF29 was a BV-specific structural protein. Further experiment also revealed that BmNPV ORF29 was present in the envelope fraction of BV.

Intracellular localization of BmNPV ORF29 protein in BmN cells

Intracellular localization of BmNPV ORF29 was determined by the immunofluorescence assay using BmNPV-infected cells and anti-BmNPV ORF29 antiserum. Since BmNPV ORF29 protein was first detected at 6 hrs p.i., we chose time points of 6, 12, and 24 hrs p.i. for examination. The results revealed that the BmNPV ORF29 was localized primarily in the cytoplasm and nucleus (Fig. 5). In control experiment no obvious fluorescence signal was observed in the BmNPV-infected cells treated with FITC-conjugated goat anti-rabbit IgG (data not shown).

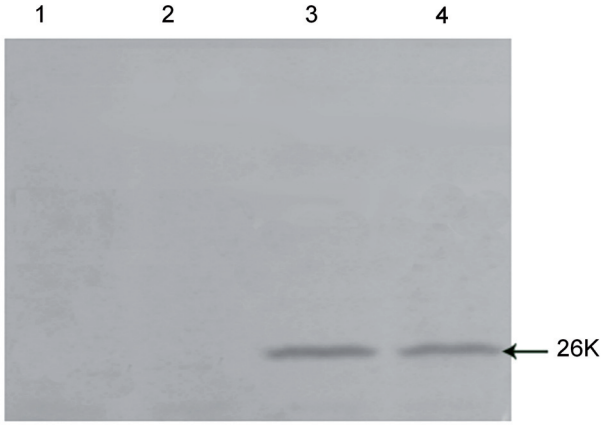

Fig. 4

Localization of BmNPV ORF29 protein in virions

Western blot analysis. Purified ODV (lane 1), BV nucleocapsids (lane 2), BV envelopes (lane 3), and BV (lane 4).

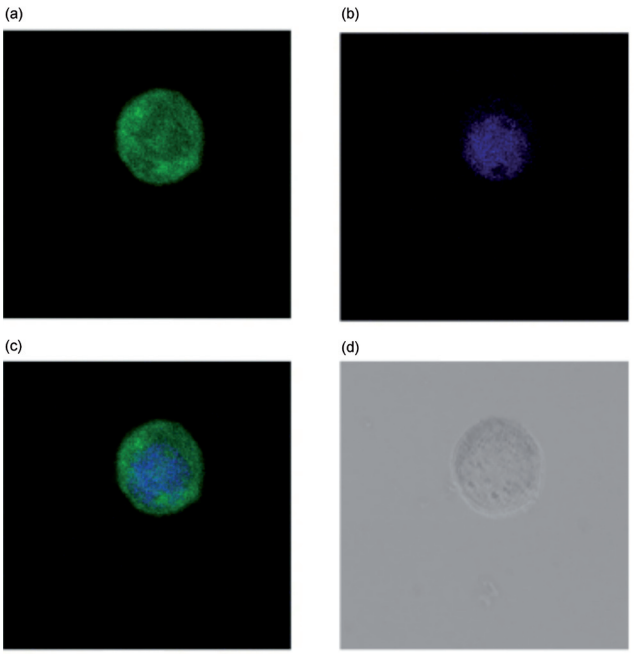

Fig. 5

Subcellular localization of BmNPV ORF29 protein in the virus-infected BmN cells.

Immunofluorescence assay. Standard staining (a), DAPI staining for nuclei (b), merged pictures a and b (c), no staining (d).

Discussion

BmNPV ORF29 is a conserved gene with homologues present in most baculoviruses infecting lepidopteran insects. The length and identity of the homologies were quite variable, but they shared the same motifs as Nudix proteins. BmNPV ORF29 and its homologues in the baculoviruses have two aa residues different from the consensus of Nudix motif, $GX_5EX_7REUXEEXGU$. The first E is replaced by D and the last residue U is replaced by R or T in BmNPV ORF29 and its homologues that are not bulky aa (U). The variations of the Nudix motif are also presented in other species, although the roles of these alterations are not clear (McLennan, 2006).

Both BmNPV ORF29 transcript and product (26 K) were detected predominantly at early stages in the infection cycle

and this finding was compatible with the observation that 26 K protein was present in BV. Thus, it is required when BV is assembled. The BmNPV ORF29 protein was associated with the BV envelope, but not with ODV suggesting that the BmNPV ORF29 protein may either play a role in the assembly of BV or relate to the BV infection of cells. In previous studies of baculovirus envelope proteins, the proteins GP64, Ld130, and Se8 are involved in the virion attachment, membrane fusion, and budding from cells (Blissard and Wenz, 1992; Pearson *et al*., 2000; WF *et al*., 2000). It has also been reported that viral envelope proteins such as GP64 and Ld130 accumulated in the plasma membranes of infected cells (Blissard and Rohrmann, 1989; Pearson *et al*., 2000). In contrast, BmNPV ORF29 was shown to localize in the cytoplasm and nucleus of the infected cells suggesting that the function of BmNPV ORF29 was different from the major envelope proteins. To understand more fully the function of BmNPV ORF29, the analysis of protein interactions and electron microscopical examination are required.

Western blots showed that Mr of translational product of BmNPV ORF29 was 26 K, what corresponded with the size deduced from the sequence of BmNPV ORF29. These results showed that no major post-translational modifications occurred in the BmNPV ORF29 protein, despite the presence of several potential modification motifs in BmNPV ORF29 aa sequence.

BmNPV ORF29 belongs to the Nudix family, which is a group of versatile and widely distributed enzymes. Substrates of the Nudix enzymes are linked to some potentially toxic, deleterious compounds such as ADP-ribose (ADPR) or some important cell signaling molecules (Dunn *et al*., 1999). In recent research, Autographa californica multiple nucleopolyhedrovirus ORF38, BmNPV ORF29 homologue, has been shown to have ADPRase activity (Du *et al*., 2007). ADPRase has been suggested to play a role in the regulation of energy metabolism, ion channel, and other important functions of the cell (Dunn *et al*., 1999). Such activities may help also to the baculovirus replication in the host cells.

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