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Helicoverpa armigera single nucleocapsid nucleopolyhedrovirus ORF51 is a ChaB homologous gene involved in budded virus production and DNA replication

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ABSTRACT

The baculovirus ChaB proteins are conserved in all completely sequenced Lepidopteran NPVs and are annotated as putative DNA binding proteins. Here we investigated *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearNPV) ORF51 (*ha51*), one of the ChaB homologues in HearNPV. 5′-RACE revealed that *Ha51* is transcribed from a conventional early promoter transcriptional initiator motif (CATT) located at 159nt upstream of ATG. RT-PCR confirmed that *ha51* is an early transcribed gene. To study the function of *Ha51* in the life cycle of HearNPV, *Ha51* knockout and repair bacmids were generated by homologous recombination in *Escherichia coli*. Growth curve and DNA replication analyses showed that the levels of budded virus (BV) production and viral DNA accumulation were significantly higher in cells infected with *Ha51* null virus than those infected with wild-type bacmid derived virus. Electron microscopy revealed that polyhedra formation was not affected by the deletion of *Ha51*. Bioassay demonstrated that the *Ha51*-deleted virus had similar oral infectivity as the wild-type and rescued virus. Western blot analyses suggested that HA51 is a component of the nucleocapsid of BV and occlusion-derived virus as well as the envelope of BV. Immunofluorescence microscopy showed that HA51 protein is mainly localized in the cytoplasm of infected cells. Taken together, our results indicate that, unlike previously characterized baculovirual ChaB genes, *Ha51* is involved in viral DNA replication and BV production and is transcribed in the early stage of infection.

1. Introduction

The *Baculoviridae* is a large family of viruses that infect arthropods, particularly Lepidoptera. Nucleopolyhedroviruses (NPVs), members of *Baculoviridae*, have a large, circular genome of supercoiled, double-stranded DNA packaged into rod-shaped virions. *Helicoverpa armigera* single nucleocapsid NPV (HearNPV) was first isolated in 1975 in Hubei Province of the People's Republic of China and has been used extensively over 25 years in China to control *H. armigera* in cotton (Zhang, 1994). The size of the HearNPV genome is 131 kb and contains 135 open reading frames (ORFs) with protein-encoding potential (Chen et al., 2001). Similar to *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), the archetype virus of the *Baculoviridae*, HearNPV produces two forms of virus progeny during its infection cycle, budded viruses (BVs) and occlusion-derived virus (ODVs). Although the two forms contain identical genetic materials, they are produced at different stages of virus infection and mediate baculovirus infection though different routes. BVs are produced during the early stage of baculovirus infection and mediate systematic infection within the host. ODVs are produced during very late stage of baculovirus infection and embedded within a crystalline structure comprised of polyhedrin proteins that form occlusion bodies. Occlusion bodies are very stable in the natural environment and are disintegrated under alkaline condition in the midgut of insects after ingestion, releasing ODVs for primary infection.

To date, at least 50 baculovirus genomes have been sequenced and analyzed. Blast search revealed that all Lepidopteran NPVs contain a pair of neighboring ORFs homologous to the bacterial ChaB protein such as HearNPV ORF51 (*Ha51*), ORF52 (*Ha52*), and AcMNPV ORF59, ORF60. In addition, two Betabaculoviruses, *Agrotis segetum* (As) GV, and *Xestia c-nigrum* (Xc) GV, also contain one such homolog.

ChaB is a putative regulator of ChaA, a Na+/H+ antiporter in *Escherichia coli* and contains a conserved 60-residue region of unknown function found in other bacteria, Archaea and a series of baculovirus proteins. To date, no ChaB domains have been identified in vertebrate and plant species. The 3D structure of ChaB has been well defined and is composed of 3 α-helices and a small sheet that packs tightly to form a fold that is found in the cyclin-box family of proteins. A tightly packed hydrophobic core between the two long helices (α2 and α3) stabilizes the overall fold of ChaB. Most

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notably, an area of negative charge is observed at the highly mobile loop 2 and the helices surrounding it (Osborne et al., 2004). ChaB proteins are classified into two major groups based on sequence alignments. Group I consists of ChaB proteins found in bacteria and Archea, while Group II contain ChaB-related proteins found in the *Baculoviridae*. The ChaB proteins found within group I are annotated as cation transport regulators based on being part of the ChaA operon. ChaB sequences belonging to group II (*Baculoviridae*) are annotated as putative DNA binding proteins. Clearly, further studies are required to determine the biological significance of baculovirus ChaB homologs for virus infection.

Previous reports have showed that *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) and *Spodoptera litura* multicapsid nucleopolyhedrovirus (SpltMNPV) ChaB proteins are transcribed at late stage of infection within the baculovirus consensus late promoter motif ATAAG, suggesting that they may function as DNA binding proteins (Li et al., 2006a,b). In the current study, we investigated the transcription map and the expression of *Ha51* gene as well as the location of its encoded protein. We further examined the effects of *Ha51* deletion on the various stages of baculovirus infection by generation of a *Ha51* knockout bacmid. We also examined the infectivity of the recombinant viruses in *H. armigera* larvae. Our data showed that *Ha51* was not essential for virus production and oral infectivity to larvae, but deletion of this gene appeared to increase the BV titer and viral DNA accumulation level of the recombinant virus in infected cells, which is different from previously characterized baculovirual ChaB genes.

2. Materials and methods

2.1. Cells, viruses and insects

The HzAM1 cell line (McIntosh et al., 1999) was maintained in Grace's supplemented with 10% fetal bovine serum (GIBCO/BRL) (O'Reilly et al., 1992). The HaBacHZ8 bacmid derived from HearNPV-G4 strain used in this study was previously described by Wang et al. (2003) and was used to produce the wild type virus. *H. armigera* insects were maintained according to Sun et al. (1998) for HearNPV occlusion body production and bioassays.

2.2. Computer-assisted sequence analysis

The HearNPV ORF51 was analyzed using software of the European bioinformatics institute server (http://www.ebi.ac.uk/) for the prediction of domains and motifs. Conceptual translation of HA51 was compared with a variety of homologous proteins obtained from the BLAST search of updated GenBank/EMBL databases. Protein alignments were carried out using ClustalW and illustrated using WebLogo 3 (Crooks et al., 2004).

2.3. 3′ and 5′ RACE and RT-PCR analysis

HzAM1 cells were infected with BVs of HearNPV-G4 at an m.o.i. of 5. Total RNA was isolated from infected or mock-infected cells at 0, 3, 6, 9, 12, 24 and 48 h post-infection (p.i.) by Trizol extraction (Invitrogen). RT-PCR was performed with 3 μg purified total RNA as template per time point. After treating with RNase-free DNase I (Promega), cDNA was synthesized by using M-MLV (Promega) and an oligo (dT) anchor primer. The coding region of the *Ha51* gene was amplified by PCR with the *Ha51*-specific primers Ha51F (5′-GGCATGTATTATCTAGACAAAATGATCG-3′) and Ha51R (5′-GGCTTAATTGCTGTCATATTCATCGC-3′). Total RNA from mock-infected cells was used as a negative control. The PCR products were analyzed on the 1% agarose gel.

To map the transcription stop sites for *Ha51*, total RNA was extracted from HearNPV-G4 infected cells by Trizol (Invitrogen). First-strand cDNA synthesis was performed using 3 μg of total RNA as template with M-MLV (Promega) and an oligo (dT) anchor primer (5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV-3′). The cDNA was amplified using an anchor primer (5′-GACCAC GCGTATCGATGTCGAC-3′) and the *Ha51*-specific primer, Ha51F. The PCR products were purified and ligated into pGEM-T vector (Promega) and sequenced to determine the 3′ end of the ORF51 transcript.

The 5′ initiation sites of the *Ha51* transcript were determined using the 5′/3′ RACE kit (Roche) with 3 μg of total RNA as template. Briefly, first-strand cDNA synthesis was performed with the gene-specific reverse primer (R1: 5′-TCTTCGTCGACGATATCGTGTCG-3′). The cDNA was purified using the High Pure PCR Purification kit (Roche) and a poly(A) tail was added to the 3′ end using terminal transferase and dATP. The tailed cDNAs were amplified by PCR using the above oligo(dT) anchor primer and the first nested, gene-specific reverse primer (R2: 5′-CGGTTTTTGGTATCCATCTGTTG-3′). A second PCR was performed using the anchor primer and the second nested primer (R3: 5′-AATGACGTGTGACTGCTTCATAGG-3′). The PCR products were purified and ligated into pGEM-T vector and sequenced.

2.4. Generation of the polyclonal antibody against HA51

The complete coding region of ORF51 (nt42412–42894) was PCR amplified using Pet51F (5′-GGGGGATCCATGTATTATCTAGACA AAAT-3′) (B*am*HI site underlined) and Pet51R (5′-GGGAAGCTT TTAATTGCTGTCATATTCAT-3′) (H*ind*III site underlined). The purified PCR product was cloned into the pET-28a(+) expression vector (Novagen), named pET-28a-HA51, and gene of interest was expressed as a His-tag fusion protein (His-tag-HA51) in *E. coli* BL21 cells. Recombinant proteins were purified according to the manufacturer's protocol (Novagen) and used to raise polyclonal antibodies in rabbits using Freund's adjuvant (Sigma).

2.5. Western blot analysis of HA51 expression

Monolayers of HzAM1 cells were mock or HearNPV-G4 infected at an m.o.i. of 5. The infected cells were harvested at 6, 12, 24, 48, 72 and 96 h p.i. and subjected to SDS-PAGE and Western blot analysis. Membranes were allowed to react with the anti-HA51 antiserum diluted 1:100. Immunoreactive proteins were visualized using goat anti-rabbit IgG conjugated with alkaline phosphatase and NBT/BCIP (Roche Appl. Sc.).

2.6. Purification of BV and ODV fractions and Western blot analysis

The envelope and nucleocapsid fractions of BV and ODV were separated after treatment with NP-40 as previously described (Deng et al., 2007). Proteins of purified BVs and ODVs, as well as their nucleocapsid and envelope fractions, were separated by 12% SDS-PAGE and transferred onto Hybond-N membranes (Millipore). Anti-HA51, anti-VP39 and anti-E18 antiserum were used for Western blot analysis for nucleocapsid, ODV envelope and BV envelope-specific proteins, respectively.

2.7. Immunofluorescence microscopy

At various times post-infection, culture supernatants were removed and HearNPV-G4 infected cells were washed three times with PBS and fixed in 2% formaldehyde in PBS/pH 7.4 for 15 min. The fixed cells were then washed three times for 15 min, followed by permeabilization in 0.2% Triton X-100 plus 1% normal goat serum in PBS for 5 min on ice. Cells were then incubated with polyclonal antibody against HearNPV-VP39 (1:1000) or HA51 (1:500) for 1 h at