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Characterization of Ha29, a Specific Gene for *Helicoverpa armigera* Single-nucleocapsid Nucleopolyhedrovirus

Zhong-Jian Guo, Shi-Heng An, Dun Wang, Yan-He Liu, V. Shyam Kumar and Chuan-Xi Zhang*
Institute of Applied Entomology, Zhejiang University, Kaixuan Road 268, Hangzhou 310029, Zhejiang, China

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Open reading frame 29 (*ha*29) is a gene specific for *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus (HearSNPV). Sequence analyses showed that the transcription factor Tfb2 motif, bromodomain and Half-A-TPR (HAT) repeat were present at aa 66-82, 4-76, 55-90 of the Ha29 protein respectively. The product of Ha29 was detected in HearSNPV-infected HzAM1 cells at 3 h post-infection. Western blot analysis using a polyclonal antibody produced by immunizing a rabbit with purified GST-Ha29 fusion protein indicates that Ha29 is an early gene. The size of Ha29 product in infected HzAM1 cells was about 25 kDa, which was larger than the presumed size of 20.4 kDa. Tunicamycin treatment of HearSNPV-infected HzAM1 cells suggested that the Ha29 protein is N-glycosylated. Fluorescent confocal laser scanning microscope examination, and Western blot analysis of purified budded virus (BVs), occlusion-derived virus (ODVs), cell nuclear and cytoplasmic fraction, showed that the Ha29 protein was localized in the nucleus. Our results suggested that *ha*29 of HearSNPV encodes a non-structurally functional protein that may be associated with virus gene transcription in *Helicoverpa* hosts.

Keywords: Expression, Ha29, *Helicoverpa armigera*; Localization, Single-nucleocapsid nucleopolyhedrovirus

Introduction

The *Baculoviridae* family is a group of enveloped double-stranded DNA viruses that are highly selective pathogens in arthropods, mainly in insects of the order Lepidoptera. *Baculoviridae* is taxonomically subdivided into two genera, the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs), based on the morphology of occlusion bodies (OBs) (Blissard *et al*., 2000). The NPVs have multiple virions present in large polyhedron-shaped OBs, whereas the GVs have a single virion with a single nucleocapsid embedded in a granular OB. The NPVs are further classified as single nucleocapsid NPVs (SNPVs) and multiple nucleocapsid NPVs (MNPVs) based on the number of nucleocapsids packed in a virion.

Baculoviruses have been extensively studied and developed as alternative to chemical insecticides in agriculture and forestry, in both natural and engineered forms (Wood and Granados, 1991), because they possess several properties as biological pest control agents, including higher efficacy in controlling insect pests and a less negative impact on the environment and nontarget species than the chemical pesticides (Nakai *et al.*, 2003). The cotton bollworm, *Helicoverpa armigera*, is a worldwide pest causing serious damage to a variety of agricultural crops such as cotton, tobacco, pepper, tomato, maize, sorghum and soybean (King, 1994). A baculovirus, *H. armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV), is a selective, highly infectious pathogen to this insect and has been extensively used for the control of this pest since the first isolation of this pathogen in Hubei province of China (Wang *et al.*, 2004). To date, the genomes of two isolates, one HearSNPV G4 with a genome of 131.4 kb (Chen *et al*., 2001) and the other HearSNPV C1 with a genome of 130.5 kb (Zhang *et al.*, 2005), have been completely sequenced.

So far, several HearSNPV genes, such as polyhedrin (Chen *et al*., 1997), late gene expression factor 2 (*lef-2*) (Chen *et al*., 1999), basic DNA-binding protein (BDBP) (Wang *et al*., 2001), Ha122 (Long *et al*., 2003), Ha94 (Fang *et al*., 2003) and BV-e31 (Wang *et al*., 2005), have been characterized. But the functions of many other genes, including genes common to all the baculovirus and unique to HearSNPV, still remain unknown. It has been reported that twenty open reading frames (ORFs), including *ha*29 were found only in HearSNPV and its closely related *H. zea* SNPV (Chen *et al*., 2001, 2002); some of which may be responsible for the

*To whom correspondence should be addressed.
Tel: 86-571-86971697; Fax: 86-571-86971697
E-mail: chxzhang@zju.edu.cn

unique features such as host specificity of this virus (Long *et al*, 2003). In this paper, we confirmed that *ha*29 is a functional gene expressed immediately during early post-infection. The *ha*29 encodes a protein that is present within the nucleus. Together with the information obtained online by motif-predicted tools, the function of Ha29 was thought to be associated with gene transcription in *Helicoverpa* host.

Materials and Methods

Insect, virus and cell line *Helicoverpa armigera* larvae were reared on an artificial diet at 28 ± 1°C, 16: 8 h light/dark photoperiod and 75% relative humidity. Wild-type HearSNPV C1 isolate was propagated in the larvae and the cell line HzAM1. The cells were cultured at 27°C in TNM-FH medium (Sigma, St. Louis, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, USA).

Computer-assisted sequence analysis Analysis of the deduced Ha29 protein sequence was carried out by MotifScan online (http://www.expasy.org/tools) (Falquet *et al*., 2002) and the Simple Modular Architecture Research Tool (SMART) (Schultz *et al.*, 1998; Letunic *et al.*, 2004) from the website http://smart.embl-heidelberg.de/ for the prediction of domains, motifs, post-translational modifications, signal sequences. Transmembrane regions were predicted by TMHMM Server v. 2.0 in the website http://www.cbs.dtu.dk/services/TMHMM. Protein comparison in the databases, GenBank and SWISS-PROT, was done with BLASTP tool.

Cloning, expression and purification of Ha29 protein The Ha29 coding region was amplified from HearSNPV C1 isolate genome by PCR, using forward primer 5'-AGGATCCATGTCCGAAATATCGCAC-3' (*Bam*HI site underlined) and reverse primer 5'-ACTCGAGTTATTCGAATCTCATCACG-3' (*Xho*I site underlined). The PCR products were ligated into pGEM-T Easy vector (Promega, Madison, USA) and sequenced using the T7 primer. The insert was retrieved by digestion with *Bam*HI and *Xho*I, and was cloned into *Bam*HI and *Xho*I sites of pGEX-4T-2, fused with glutathione S-transferase (GST) in this plasmid. The produced plasmid was named pGEX-GST-Ha29.

The vector pGEX-GST-Ha29 was transformed into *Escherichia coli* BL21 (DE3) LysS cells (Novagen, Madison, USA) and fusion protein expression was induced by 0.15 mM isopropyl β-D-thiogalactoside (IPTG; Sigma) in the presence of ampicillin at 27°C overnight. Bacterial cells were harvested at 6000 × *g* for 15 min at 4°C, and were lysed in Laemmli protein sample buffer (Laemmli, 1970) by boiling for 5 min. The proteins were electrophoresed on vertical polyacrylamide (10%) slab gel as described by Sambrook and Russell (2002) and the bands were visualized by staining with Coommassie brilliant blue R250.

The highly expressed GST-fused protein was purified using the Glutathione Sepharose 4B according to the method described in the GST Gene Fusion System Manual (Amersham Pharmacia Biotech).

Production of polyclonal antiserum New Zealand white rabbit was injected 4 times, with 80 μg purified GST-Ha29 fusion protein in one milliliter sterilized water supplemented with 20 μg/ml streptomycin and 40 U penicillin per each dose. The first immunization was performed hypodermically in 50% Freund's complete adjuvant followed by the second injection intramuscularly in 50% Freund's incomplete adjuvant with 3-week interval, according to the protocol described earlier (Harlow and Lane, 1988). Two weeks after the second injection the rabbit was immunized third time with purified GST-Ha29 fusion protein. One week later, the last injection of purified GST-Ha29 fusion protein was given to boost immunization. The rabbit was exsanguinated 1 week after the last boost. Enzyme-linked immunosorbent assay (ELISA) using *E. coli* BL21 cell extracts expressing GST-Ha29 and column-purified GST-Ha29 was used to test the activity and specificity of the polyclonal antiserum.

Immunodetection of the Ha29 protein expressed in insect host cells Monolayers of HzAM1 cells were infected with wild-type HearSNPV C1 at an m.o.i. of approximately 10 $TCID_{50}$ units per cell and incubated at 27°C. Cells were harvested at 0, 3, 6, 12, 24, 48 and 72 h p.i., washed twice in 1× phosphate-buffered saline (PBS) and lysed in SDS-PAGE loading buffer by boiling for 5 min to completely denature the proteins. The cellular debris was removed by centrifugation at 12,000 rpm for 10 min. The samples were separated by SDS-PAGE using 10% polyacrylamide and analyzed by Western blot (Sambrook and Russell, 2002).

Purification of HearSNPV BV and ODV fractions HzAM1 cells were infected with BVs of HearSNPV C1 strain at an m.o.i. of 10 $TCID_{50}$ units per cell. Three days later, purification of BVs was done as described by Long *et al.* (2003). Polyhedral inclusion bodies (PIBs) were isolated from infected *H. armigera* fifth-instar larvae and purified by sucrose gradient centrifugation (IJkel *et al.*, 2000). Occlusion-derived viruses (ODVs) were isolated from PIBs as described previously (Shippam *et al.*, 1997). Fractionating of the BV virions into envelope and nucleocapsid fractions after treatment with Nonidet P-40 was done as described by IJkel *et al*. (2000).

Nuclear and cytoplasmic fractions of virus-infected HzAM1 cells Nuclear and cytoplasmic fractionation was performed as described earlier (Jarvis *et al*., 1991; Shippam *et al.*, 1997). Briefly, the adherent monolayer cells in a 35 mm diameter culture dish were infected as described earlier. After 3 days, the dish was washed twice with 1× PBS, followed by adding 0.5 ml of cell lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 5 mM $MgCl_2$, 0.5% v/v Nonidet P-40) containing 1× protease inhibitor cocktail (50 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). The dish was placed on ice for 30 min by occasional mixing and the nuclei was pelleted at 1,000 × *g* for 5 min. The supernatant was removed and Laemmli protein sample buffer was added. The nuclear pellet was resuspended in cell lysis buffer and Laemmli protein sample buffer was added. The total cell proteins were prepared by adding cell lysis buffer and Laemmli protein sample buffer to the whole cells.

Confocal laser scanning microscope HzAM1 cells were grown in a plastic petri dish and infected with wild-type HearSNPV. At 48 h p.i., cells were washed three time in 1× PBS, fixed with 4% paraformaldehyde in 1× PBS followed by permeating with 0.2%

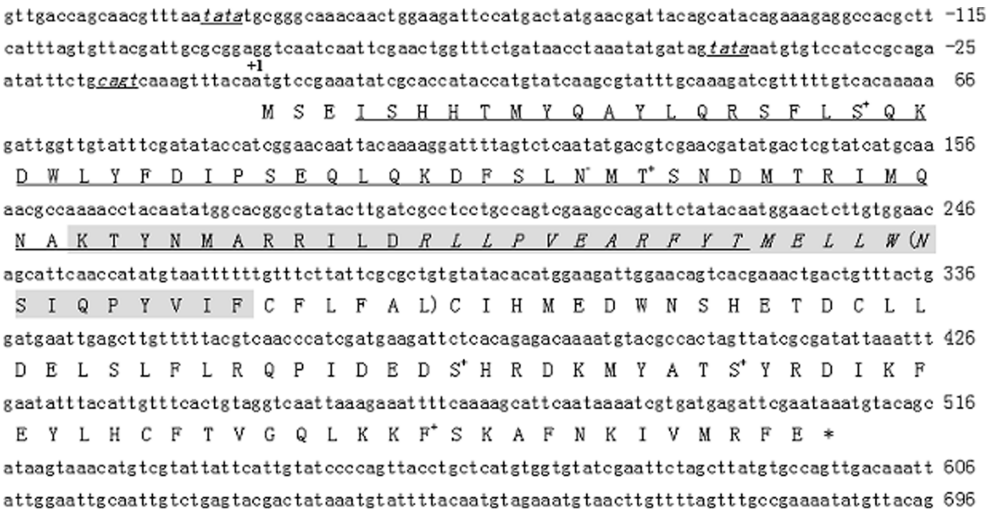

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *ha*29 region from 25147 to 25653 bp in HearSNPV C1 genome (GenBank accession number AF303045). The motifs were deduced by several search engines online. Superscript +s indicate the putative phosphorylated amino acids. The third aa of the cAMP- and cGMP-dependent protein kinase phosphorylation site [RK]-x-[S/T], the first aa of the casein kinase II phosphorylation site [S/T]-xx-[D/E], and the first aa of the protein kinase C phosphorylation site [S/T]-x-[R/K] are phosphorylated respectively. Superscript-suggests the putative N-glycosylation site N-x-[S/T]-x. Locations of two TATA boxes, baculovirus consensus early transcriptional start motif CAGT are in italics and underlined. The deduced transmembrane region is shown in brackets. The putative bromodomain is underlined. The HAT repeat and transcription-factor-Tfb2-likely motifs are shown in shadow and italics respectively.

Triton X-100 in 1× PBS for 10 min, and allowed to react with the obtained polyclonal antiserum diluted 1 : 5000 and immunoglobulin G fused with enhanced green fluorescent protein (EGFP), according to the protocol described previously (Spector *et al.*, 1998). Following treatment the cells were examined under a Zeiss LSM510 confocal laser-scanning microscope for fluorescence detection.

N-glycosylation assay HzAM1 cells were infected with wild-type HearSNPV C1 in presence of 10 μg/ml tunicamycin. Cells were harvested by centrifugation at 72 h p.i. and analyzed by Western blot.

Results

Sequence analysis of *ha*29 The *ha*29 gene was 507 nucleotides long, potentially encoding a protein of 168 amino acids (aa) with a predicted molecular weight of 20.4 kDa. Two TATA boxes were found at 185 and 46 nts upstream of the ATG codon. A baculovirus consensus early transcriptional start motif, CAGT was also observed at 15 nts upstream of the ATG (Fig. 1). These analyses suggested that *ha*29 was most likely an early gene.

Search in protein databases, GenBank and SWISS-PROT, showed that the putative Ha29 protein was only found in HearSNPV and *Helicoverpa zea* single-nucleocapsid nucleopolyhedrovirus (HezeSNPV, a possible variant of HearSNPV) with no homologues in other baculoviruses.

A transmembrane region at aa 82-98 was observed by the search engine TMHMM Server v. 2.0 in the website http://www.cbs.dtu.dk/services/TMHMM/. Using MotifScan Server in the website http://myhits.isb-sib.ch/cgi-bin /motif_scan, eight putative phosphorylation sites were found in the putative Ha29 protein (Fig. 1), including four casein kinase II phosphorylation sites (aa 20-23, 43-46, 127-130, 136–139), three protein kinase C phosphorylation sites (aa 20-22, 127-129, 136-138) and one cAMP- and cGMP-dependent protein kinase phosphorylation site (aa 154-157). A putative N-glycosylation site was also found at aa 41-44. Another significance at aa 66-82 was the transcription- factor-Tfb2-likely motif. It was reported that the transcription factor Tfb2 was a family whose members were parts of the TFIIH complex involved in the DNA-dependent initiation of transcription and nucleotide excision repair (Orphanides *et al.*, 1996). Using the Simple Modular Architecture Research Tool (SMART) (Schultz *et al.*, 1998; Letunic *et al.*, 2004) in the website http://smart.embl-heidelberg.de/, a bromodomain and a Half-A-TPR (HAT) repeat were observed at aa 4-76 and aa 55-90 respectively (Fig. 1).

Immunodetection of the Ha29 protein in infected cells To study the possible function of Ha29 protein, a polyclonal rabbit antibody was prepared. At the beginning, the Ha29 protein was expressed in *E. coli* in frame with GST tag and purified by column chromatography (Fig. 2). The size of the purified GST-Ha29 fusion protein was about 38 kDa, smaller than theoretical size of 46.4 kDa. But this exerted little influence on antiserum generation. Immunization of rabbit with the purified GST-Ha29 fusion protein was performed to generate anti-Ha29 antiserum.

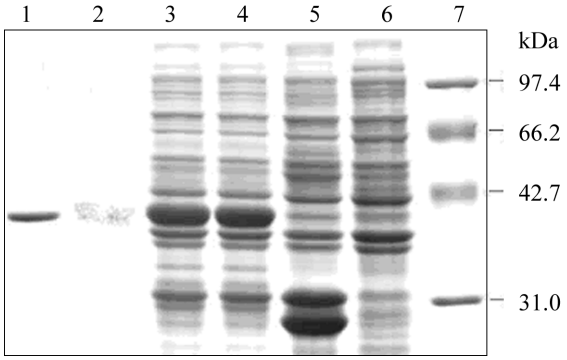

Fig. 2. Expression of the Ha29 protein in *E. coli* BL21 (DE3) LysS cells. BL21 cells transformed with pGEX-GST-Ha29 fusion plasmid were induced by IPTG and the cell extracts were separated by SDS-PAGE. Lane 1, purification of the GST-Ha29 fusion protein by column chromatography; lane 2, Western blotting analysis using anti-GST antibody; lane 3 and 4, bacterial proteins from BL21 cells transformed with pGEX-GST-Ha29 fusion plasmid induced by IPTG; lane 5, proteins from BL21 transformed with pGEX-4T-2 plasmid; lane 6, BL21 cell proteins with no plasmid transformed, and lane 7, protein marker.

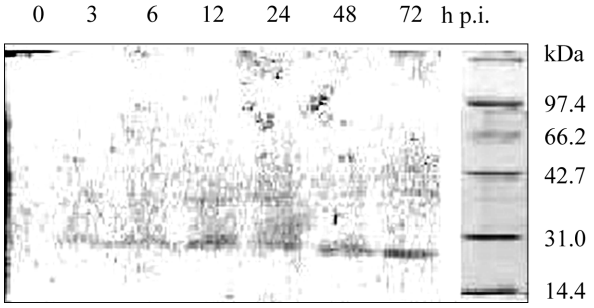

Fig. 3. Western blot analysis of the Ha29 protein in HearSNPV-infected HzAM1 cells. Cells were collected from 0 to 72 h p.i. The total cell denatured proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and reacted with anti-Ha29 polyclonal anti-rabbit serum.

A time course analysis was done to detect the Ha29 protein expressed in HearSNPV-infected HzAM1 cells, using the anti-Ha29 rabbit antiserum. A faint band was first detected at 3 h p.i., indicating that the Ha29 protein was synthesized soon after infection (Fig. 3). This was in accordance with the DNA sequence analysis. The band of about 25 kDa in size larger than predicted molecular mass of 20.4 kDa was observed, suggesting that the Ha29 protein was modified after translation.

Localization of the Ha29 protein in cell, BV and ODV To investigate whether the Ha29 protein was a structural protein, Western blot analysis of purified BVs, ODVs and HearSNPV-infected cell fractionations were done for immunodetection (Fig. 4A-C). A clear band was detected in the sample of nuclear fraction. In contrast, no band was observed in the cytoplasmic fraction, BV and ODV samples, indicating that

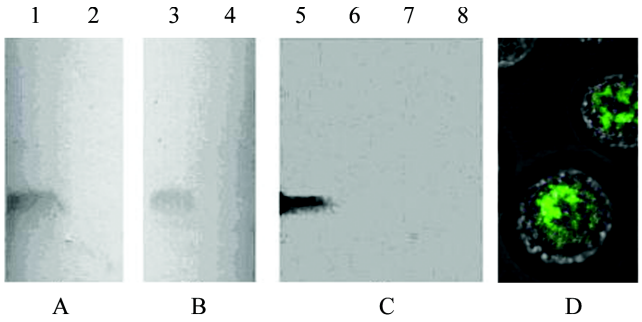

Fig. 4. Localization of Ha29 protein in HearSNPV C1 BVs, ODVs and HzAM1 cells. BV, ODV, cell nuclear and cytoplasmic fraction samples were separated by SDS-PAGE and analyzed by Western blotting. (A) Cells harvested at 6 h p.i.; (B) Cells harvested at 24 h p.i.; (C) Western blot analysis of Ha29 protein in BV, ODVs and total cell proteins. Lane1, 3, cell nuclear fraction; lane2, 4, cell cytoplasmic fraction; lane 5, total proteins of infected cells; lane 6, proteins of uninfected cells; lane 7, purified ODV and lane 8, purified BV. (D) Immunofluorescent examination under a Zeiss LSM510 confocal laser scanning microscope. Ha29 protein was located in the enlarged nuclei of the cells infected with HearSNPV.

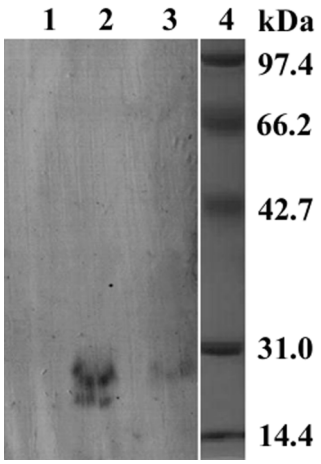

Fig. 5. Western blotting to analyze N-glycosylation of Ha29 protein in HearSNPV-infected HzAM1 cells. Lane 1, protein sample harvested at 0 h p.i.; lane 2, protein sample exposed and not exposed (lane 3) to 10 μg/ml tunicamycin, an inhibitor of N-glycosylation; lane 4, protein marker.

the product of *ha*29 was a non-structurally functional protein. Confocal laser scanning microscope detection showed that the Ha29 protein was localized in nucleus (Fig. 4D), suggesting that the Ha29 protein was correlated to the events happening in the nucleus.

Glycosylation assay of Ha29 In order to determine whether the Ha29 protein was glycosylated, HearSNPV-infected HzAM1 cells were exposed to an inhibitor of N-glycosylation, tunicamycin. Immunoblotting revealed that tunicamycin treatment of infected cells resulted in the appearance of two distinct bands of about 25 kDa and 22 kDa in size (Fig. 5). In

contrast, a single band of about 25 kDa was observed on condition that tunicamycin was absent from cell culture medium. The result indicated that the Ha29 protein was N-glycosylated.

Discussion

During the present study we have described a novel nonstructural protein encoded by HearSNPV ORF29. Like Ha122 (Long *et al.*, 2003), this protein is unique to HearSNPV and HezeSNPV, and no its homologues were found in any other baculovirus to date. Potentially, the coding region of *ha*29 encodes a 20.4 kDa protein, which is smaller than the size of 25 kDa as determined by SDS-PAGE (Fig. 3), suggesting that the Ha29 protein is post-translationally modified, such as phosphorylation and/or glycosylation. When the cells were treated by tunicamycin, an inhibitor of N-glycosylation, two distinct bands were observed, indicating that the Ha29 protein is N-glycosylated undoubtedly.

The Ha29 protein is detected during early stages of the infection cycle and this conforms to the sequence analysis that there is a baculovirus consensus early transcriptional start motif CAGT preceded by two TATA boxes upstream of the ATG codon. The *ha*29 is an early gene. It is reported that early genes play a critical role in temporally regulating gene expression in the baculovirus (Blissard and Rohrmann, 1990; Friesen, 1997). The Ha29 protein was not detected in the ODV, BV and their envelope and nucleocapsid fraction treated with Nonidet P-40 (Data not shown), but was detected in the cell nuclear fraction, suggesting that the product of Ha29 is a non-structurally functional protein. Fluorescent confocal laser scanning microscope examination further confirmed that the Ha29 product was localized in the nucleus. Additionally, the Ha29 protein was also found on the karyotheca (data no shown). This may be correlated to the transmembrane region at aa 82-98 deduced by TMHMM Server v. 2.0 engine.

Using the Simple Modular Architecture Research Tool (SMART) (Schultz *et al.*, 1998; Letunic *et al.*, 2004) in the website http://smart.embl-heidelberg.de/, a bromodomain and Half-A-TPR (HAT) repeat were found at aa 4-76 and aa 55-90 respectively. The bromodomains were observed in a variety of mammalian, invertebrate and yeast DNA-binding proteins (Haynes *et al.*, 1992). The bromodomains could interact specifically with acetylated lysine (Jeanmougin *et al.*, 1997; Dhalluin *et al.*, 1999). Histone acetylation plays an important role in chromatin remodelling and gene activation (Carey and Smale, 2000). Nearly all known histone- acetyltransferase-associated transcriptional co-activators contain bromodomains, which are found in many chromatin-associated proteins (Dhalluin *et al.*, 1999). In some proteins, the classical bromodomain has diverged to such an extent that parts of the region are either missing or contain an insertion. The bromodomain may occur as a single copy, or in duplicate. In all, though the precise function of this domain remains unclear, but it may be involved in protein-protein interactions and may play a role in assembly or activity of multi-component complexes involved in transcriptional activation (Tamkun, 1995). The HAT repeat has a repetitive pattern characterized by three aromatic residues with a conserved spacing. They were believed to be similar to tetratricopeptide repeats (TPRs) in structure and sequence, and were found in several RNA processing proteins. The number of HAT repeats found in different proteins varied between 9 and 12. HAT-repeat-containing proteins appear to be components of macromolecular complexes that are required for RNA processing, or may be involved in protein-protein interactions (Preker and Keller, 1998). Another putative motif at aa 66-82 obtained by the MotifScan search engine was the transcription-factor-Tfb2-likely motif. It was reported that the transcription factor Tfb was a family whose members were parts of the TFIIH complex involved in the DNA-dependent initiation of transcription and nucleotide excision repair (Orphanides *et al.*, 1996). To sum up, the function of Ha29 may be associated with virus gene transcription in *Helicoverpa* hosts.

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