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Characterization of *Helicoverpa armigera* nucleopolyhedrovirus *orf33* that encodes a novel budded virion derived protein, BV-e31

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Summary. Homologues of *Helicoverpa armigera* nucleopolyhedrovirus (HearSNPV) *orf33* are found in all 22 completely sequenced members of the lepidopteran nucleopolyhedroviruses and granuloviruses, but so far their functions are unknown. In this report, we describe the characterization of HearSNPV *orf33* (*ha33*). Northern blot analysis showed a single transcript of *ha33* of approximately 0.7 kb was transcribed beginning at 3 h post-infection in infected *Helicoverpa zea* cells (HzAM1) and the gene product could be detected as early as 6 h post-infection by western blot analysis using a rabbit derived polyclonal antibody, suggesting it was an early gene. Western blot analysis also demonstrated the *ha33* protein in infected cells was a 31 kDa protein, larger than the theoretical size of 28.4 kDa, and located in the envelope fraction of budded virions (BVs). The results suggested that HearSNPV *ha33* gene is a functional gene that encodes a novel structural protein of baculovirus BVs, BV-e31.

Introduction

Baculoviruses have circular, double-stranded DNA genomes which are large enough to accommodate over a hundred genes. The family *Baculoviridae* is a large family of viruses that infect invertebrates, particularly insects of the order Lepidoptera, and is a natural control agent of insect pests in agriculture and forestry. The family has been divided into two genera on the basis of occlusion body morphology: the Nucleopolyhedroviruses (NPV), which have large occlusion bodies containing numerous virions, and the Granuloviruses (GV), which have single virions occluded within small granular occlusion bodies [23, 26].

Helicoverpa armigera single nucleocapsid nucleopolyhedrovirus (HearSNPV) belongs to the NPV genus. Baculoviruses are used widely as bioinsecticides to control heliothine insects in cotton and vegetable crops around the world [5, 12]. Two isolates of HearSNPV, C1 and G4, have been sequenced. The HearSNPV isolate G4 has a DNA genome of 135 kb [3]. We sequenced the C1 genome [29] which is 130,759 bp in length with 136 ORFs and shares 98% identity in nucleotide sequence with G4 genome. HearSNPV ORF33 (*ha33*) is located between nts 27,566 and 28,282 in the HearSNPV-C1 genome, encodes a putative 238 aa protein with predicted molecular weight of 28.4 kDa, and is transcribed in the opposite orientation of the polyhedrin gene. Homologs of *ha33* have been identified in 21 completely sequenced lepidopteran NPVs and GVs, but are not present in dipteran and hymenopteran baculoviruses. Thus far it is uncertain whether *ha33* and its homologs are functional genes. In this study, we analysed the *ha33* gene of HearSNPV isolate C1 by examining transcription of the gene and expression of its protein product in HearSNPV infected HzAM1 cells, finding that the ha33 is indeed expressed and that the ha33 protein is located on the envelope of budded virus (BV).

Materials and methods

Viruses, insects and cell lines

HearSNPV C1 was used for infections and was propagated in the *Helicoverpa zea* cell line, HzAM1 [17]. HzAM1 cells were grown in TNM-FH Insect Medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, BRL). A culture of *H. armigera* larvae was maintained according to Sun et al. [24] for HearSNPV polyhedra production. Routine methods for baculovirus manipulations were as described by O'Reilly et al. [18].

Computational analysis

The *ha33 gene* and its deduced amino acid sequence were analyzed using the Genetyx-Win (Version 5) (Software Development Co., Tokyo, Japan) and DNASTAR programs (Ver. 5.02) (DNASTAR, Inc., Madison, USA). The predictions of domains, motifs, signal sequence and post-translational modifications were carried out by ScanProsite, Motifscan, SignalP and NetPhos online (http://ca.expasy.org). Protein comparisons with entries in the updated GenBank/EMBL, SWISS-PROT and PIR databases were performed with BLASTP and PSI-BLAST programs [1] and sequence alignment was performed with Clustal-W (http://www.ebi.ac.uk/clustalw/) and edited by GeneDoc (Ver. 2.04) (Free Software Foundation, Inc., MA, USA).

Transcription analysis

Total RNA was isolated from HzAM1 cells infected with HearSNPV strain C1 (m.o.i. of 5 $TCID_{50}$ units per cell) at 0, 3, 6, 12, 24, 48 and 72 h p.i. with Trizol (Life Tech., Invitrogen, CA, USA) according to manufacturer's suggested protocol. The RNA concentration was determined by the absorbance at 260 nm. Radioactively labeled probes were prepared from the Prime-a-Gene Labeling System Kit (Promega, Madison, WI, USA), according to manufacturer's suggested protocol. RNA was denatured by heating at 65.8 °C for 15 min. For Northern blot analysis, 10 μg of total RNA (each lane) were separated by denaturating gel electrophoresis in 1% agarose/formaldehyde gels with MOPS buffer (25 mM MOPS, 5 mM sodium acetate, 2 mM EDTA). After transfer to a nylon membrane, RNA was

UV cross-linked to the membrane using a TL-2000 Ultraviolet Translinker (UVP, USA). The membranes were prehybridized in ULTRAhyb hybridization Buffer (Ambion, USA) for 3 h at 42 °C. The [^{32}P]-dCTP labeled probe was added to the hybridization solution and incubated at 42 °C for 18 h. The membranes were washed two times in 2 × SSC, 0.1% SDS at 42 °C, one time in 0.1 × SSC, 0.1% SDS at 42 °C followed by exposure to X-ray film.

Generation of anti-ha33 antiserum

The complete HearSNPV *orf33* (717 nt) was amplified by PCR using an upstream primer (5′-AGGATCCATGCGGTGCTCGGGTCTT-3′) incorporating a *Bam*HI site (underlined) and a downstream primer (5′-ACTCGAGTTAGTATTGCCGCTGCAT-3′) with an *Xho*I site (underlined). The amplified fragment was inserted into pGEM-T easy vector (Promega, USA), retrieved by digestion with *Bam*HI and *Xho*I, and subcloned into the expression vector pGEX-4T-2 (Pharmacia, USA), in-frame with the C-terminal glutathione S-transferase (GST) tag in this plasmid. The recombinant plasmid, designated pGST-*ha33*, was transformed into *Escherichia coli* BL21 cells and fusion protein expression was induced by incubation in the presence of 2 mM IPTG when the optical density of the culture at 600 nm reached ∼0.7. The GST-*ha33* fusion protein was confirmed with the mouse monoclonal anti-GST antibody (Amersham Pharmacia, USA). The recombinant *ha33* protein was purified using a GST binding column (Amersham Pharmacia, USA) and used as an immunogen to raise *ha33*-specific antiserum in male rabbits. Each rabbit received five injections of 200 μg of purified GST-*ha33* fusion protein, the first in complete Freund's adjuvant (Sigma, USA) and the second in incomplete Freund's adjuvant (Sigma, USA) followed by booster injections on days 35, 49 and 63. Ten days after the last injection, blood was recovered by cardiac puncture.

Western blot analysis

Monolayers of HzAM1 cells infected with HearSNPV-infected at an m.o.i. of 5 $TCID_{50}$ units per cell were harvested at 0, 3, 6, 12, 18, 24 and 36 h p.i., pelleted at 5000 g, resuspended in PBS and lysed in SDS-PAGE loading buffer by boiling for 10 min. Protein samples were separated by SDS-PAGE and transferred onto an Immobilon-P nitrocellulose membrane (Millipore, USA) using a semi-dry transfer cell (Trans-Blot SD, Bio-RAD, USA) according to manufacturer's suggested protocol. The membrane was incubated overnight in 2% skim milk powder in TBS (150 mM NaCl, 10 mM Tris, pH8.0) at 4 °C. The membrane was allowed to react with *ha33* antiserum diluted 1:5000 for 1 h at room temperature and subsequently processed as described by Ijkel et al. [13]. Immunoreactive proteins were visualized using goat anti-rabbit IgG-HPR (Sourthern Biotech, AL, USA) following the protocol provided by the manufacturer.

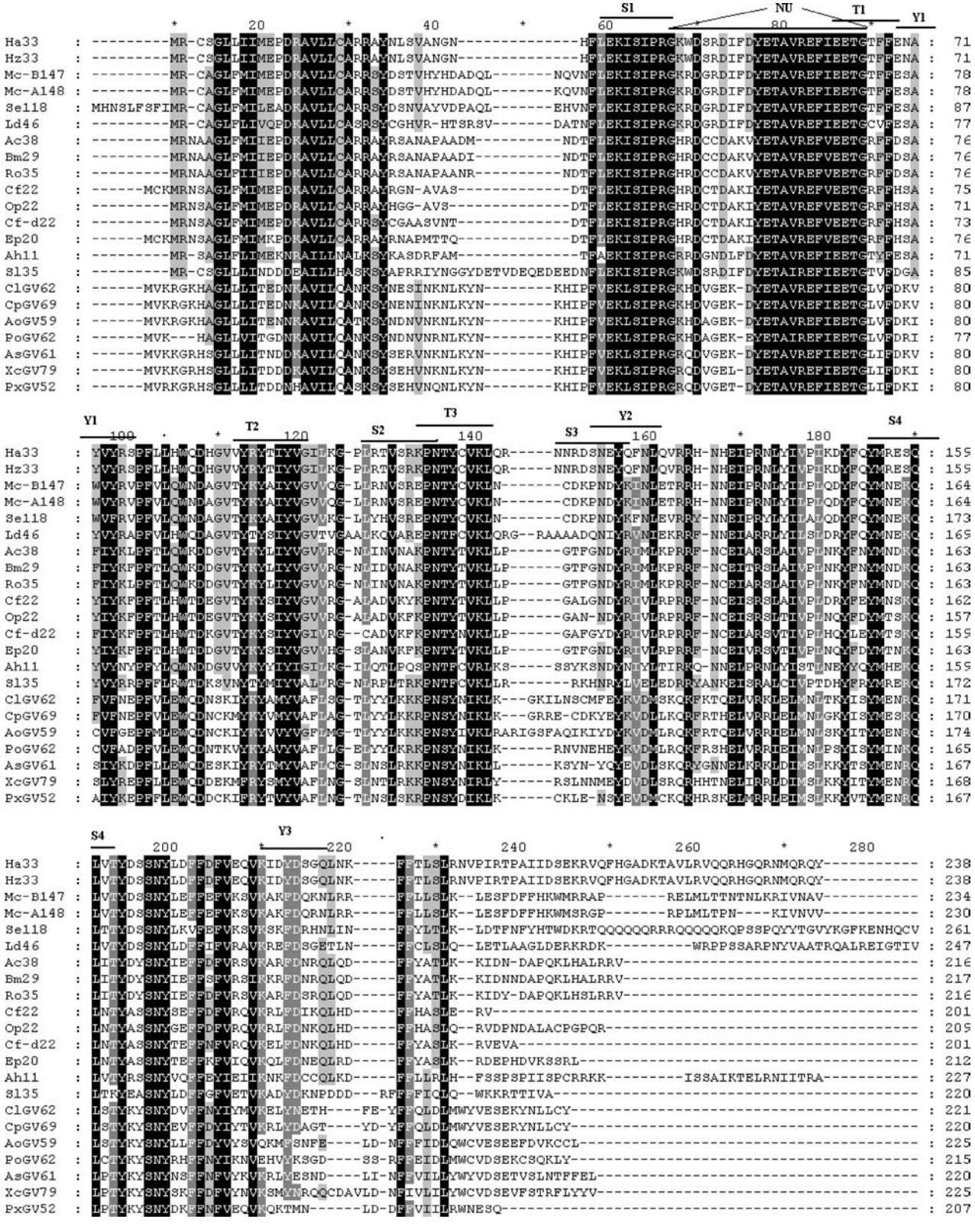
Purification of HearSNPV ODV and BV fraction

BV was purified from harvested supernatants of infected cells and ODV was purified from polyhedra as described by O'Reilly et al. [18]. For analysis of structural proteins, after purification BV's were incubated with 1% Nonidet-P40 and fractions containing the envelope and capsid were separated by centrifugation [10].

Results

Sequence analysis

The HearSNPV *ha33* gene is 717 nucleotides (nt) long, encoding a predicted protein of 238 aa with a predicted size of 28.4 kDa. A baculovirus consensus early


S1
NU
T1
Y1
Y2
T2
T3
S2
S3
S4
Y3
Ha33
Hz33
Mc-B147
Mc-A148
Se118
Ld46
Ac38
Bm29
Ro35
Cf22
Op22
Cf-d22
Ep20
Ah11
Sl35
ClGV62
CpGV69
AoGV59
PoGV62
AsGV61
XcGV79
PxGV52

promoter motif AACAGT and a TATA box are present 139 and 127 nts upstream of the start codon (ATG), respectively. This putative early promoter is completely consistent with the arthropod initiator cap site consensus [A(A/C/T)CA (G/T)T] [4].

Ten putative phosphorylation sites were found in the putative *ha33* protein including four serine phosphorylation sites (aa 59–67, 127–135, 148–156 and 184–192), three threonine phosphorylation sites (aa 86–94, 112–120 and 131–139), and three tyrosine phosphorylation sites (aa 92–100, 151–159 and 209–216) (Fig. 1). One conserved domain (residues 54–77, noted as "NU" in Fig. 1) related to the family Nudix was detected. No signal peptide, transmembrane regions, mitochondrial targeting sequences, nuclear localization signals or membrane retention signals were found by any of the motif search engines employed.

Searches of protein databases, GenBank and SWISS-PROT, revealed that the deduced *ha33* protein was homologous to the putative products from 14 NPV ORFs and seven GV ORFs (Table 1). Alignment of *ha33* sequence with its homologs from 14 NPVs and seven GVs is shown in Fig. 1. The *H. zea* NPV (a possible variation of HearSNPV) *orf33* shared an identity of 100% with HearSNPV *ha33*. The homologs from the other 13 NPVs shared 44–55% identity with HearSNPV *ha33* and the HearSNPV *ha33* was 31–35% identical to those from the seven GVs (Table 1).

Transcriptional analysis

Temporal regulation of the *ha33* transcript was examined by Northern blot analysis. This analysis also served to provide information about the number and sizes of *ha33* transcripts. A single transcript of approximate 0.7 kb was detected at 3 h p.i. and remained detectable until 48 h p.i. (Fig. 2). The transcript size of 0.7 kb is compatible with predicted ORF size of 717 nt. The detection of the transcript as early as 3 h p.i. suggested that the *ha33* gene is transcribed early in infection.

Time course of ha33 expression in HearSNPV-infected cells

In order to study the expression of the *ha33* protein and its possible function, a polyclonal antibody was prepared by immunization of rabbits with a purified GST-*ha33* fusion protein. A time course of HearSNPV-infected HzAM1 cells were analyzed by western blot using the polyclonal antibody against *ha33*. A specific immunoreactive band of approximately 31 kDa was first observed at

Fig. 1. Sequence alignments of ha33 and its homologs. Black shading: 100% identity. Grey shading: 80% identity. The putative threonine, tyrosine, and serine phosphorylation sites are denoted T(1–3), Y(1–3), and S(1–4), respectively. The conserved domain related to the family nudix is denoted with NU

Table 1. Homologous ORFs of *ha33* among baculovirus genomes

NPV/GV	Homologues	Amino acid identities (%)	GenBank accession
NPV	*Helicoverpa zea* NPV *orf33*	100	AAL56178
	Mamestra configurata NPV-B *orf147*	55	AAM95133
	Mamestra configurata NPV-A *orf148*	55	AAQ11167
	Lymantria dispar NPV *orf46*	50	AAC70231
	Spodoptera exigua orf118	49	AAF33647
	Adoxophyes honmai NPV *orf11*	48	NP_818658
	Orgyia pseudotsugata NPV *orf 22*	47	AAC59021
	Rachiplusia ou NPV *orf35*	46	AAN28089
	Choristoneura fumiferana NPV *orf 22*	46	NP_848334
	Spodoptera litura NPV *orf 35*	46	NC_003102
	Autographa californica NPV *orf 38*	45	AAA66668
	Epiphyas postvittana NPV *orf 20*	45	AAK85584
	Bombyx mori NPV *orf29*	44	NP_047444
	Choristoneura fumiferana defective NPV *orf 22*	44	AAQ91717
GV	*Agrotis segetum* GV *orf61*	35	YP_006283
	Xestia c-nigrum GV *orf79*	35	AAF05193
	Cydia pomonella GV *orf69*	33	AAK70729
	Plutella xylostella GV *orf52*	33	AAG27350
	Cryptophlebia leucotreta GV *orf62*	32	NP_891909
	Phthorimaea operculella GV *orf62*	32	AAM70260
	Adoxophyes orana GV *orf59*	31	AAP85696

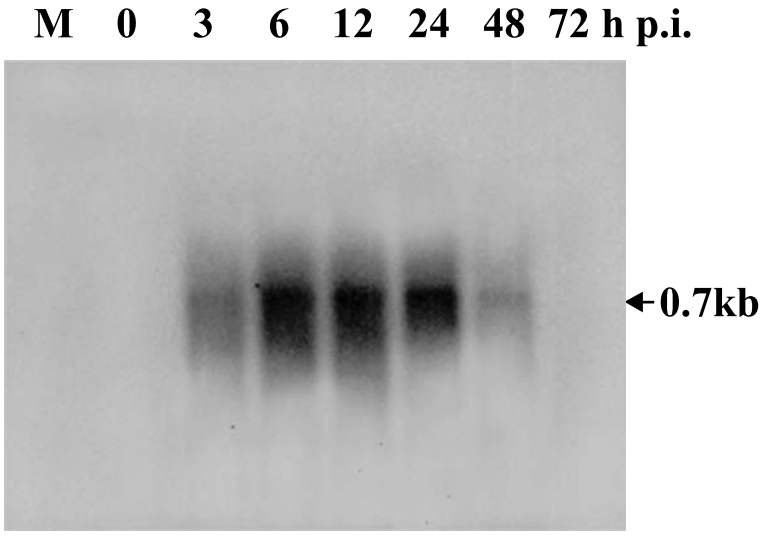


Fig. 2. Northern blot analysis of temporal regulation of transcription for *ha33* gene performed on total RNA extracted from HearSNPV-infected HzAM1 cells. The size of specific hybridization bands is indicated on the right with an arrow. Times p.i. are indicated above the lanes (*M*, mock infected)

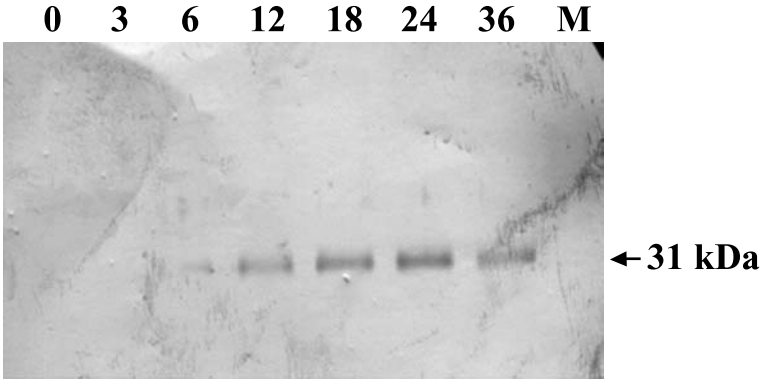


Fig. 3. Time course of *ha33* product by western blot analyses. HearSNPV-infected HzAM1 cells were harvested at the indicated times post infection. *M* mock infection. The estimated size of detected bands is indicated on the right with an arrow

6 h p.i. and remained detectable up to 48 h p.i. (Fig. 3). This was consistent with the transcriptional analysis of the *ha33* gene, however, the protein was detectable 3 hours later than the gene transcript (Fig. 2), was probably due to the low amount of protein produced shortly after transcription. Similar to the transcriptional analysis, the detection of the ha33 protein by 6 h p.i. indicated that it was synthesized early in infection.

Immunodetection of the ha33 protein in BV and ODV

To investigate whether the ha33 protein is a structural protein, western blot analysis was carried out on HearSNPV BVs and ODVs (Fig. 4). The 31 kDa protein

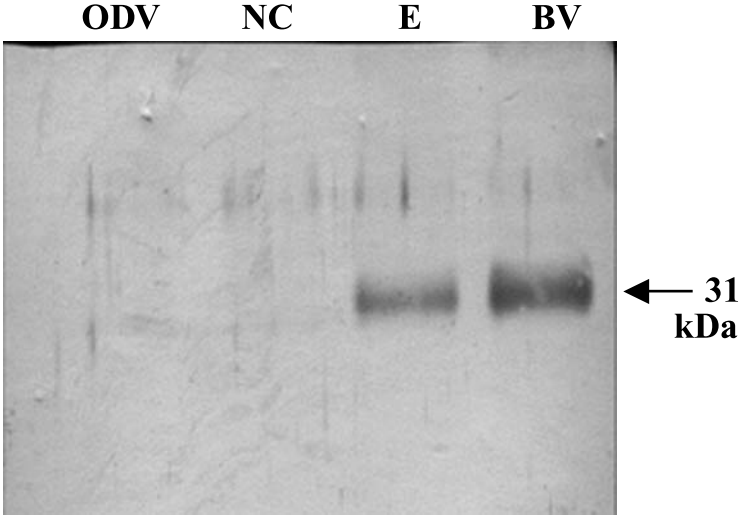


Fig. 4. Immunodetection of the ha33 protein in HearSNPV virions. ODV and BV as well as BV nucleocapsid (*NC*) and envelope (*E*) fractions were analyzed by western blot. The size of the ha33 protein is shown on the right with an arrow

of *ha33* was easily detected in a preparation of BVs (Fig. 4, lane BV). In contrast, no band could be detected in ODVs (Fig. 4, lane ODV) or in polyhedra (data not shown), indicating that ha33 was a BV-specific structural protein. Western blot analysis of virion fractions revealed that ha33 protein was present in the envelope (Fig. 4, lane E), but not in the nucleocapsid fraction of BV (lane NC).

We noticed that ha33 protein detected in BVs or infected cells was larger than the predicted molecular weight (28.4 kDa) suggesting that it might contain post-translational modifications such as phosphorylation.

Discussion

In this report, we presented transcriptional and expressional analysis of the *ha33* gene of HearSNPV, a gene that has thus far not been characterized for any baculovirus. The *ha33* gene was expressed as an early gene and encoded a novel structural protein of BV's. Northern blot analysis indicated that *ha33* transcript remained stable until 48 h p.i. (Fig. 2). This suggested that the early nature of the AACAGT promoter did not exclude transcription at time points late in infection. Similar transcription patterns have been observed for other baculovirus early genes, such as the SeMNPV Se116, Se117 genes, AcMNPV *pnk/pnl* and *lef4* genes, the BmNPV *bro* genes, and the ie1 genes of AcMNPV, OpMNPV, and LdMNPV [7, 8, 13, 16, 21, 22, 25].

Western blot analysis detected a 31-kDa structural BV-specific ha33 protein commencing at 6 h p.i. (Fig. 3). This was expected because the *ha33*-specific transcript was detected as early as 3 h p.i. (Fig. 2). These results indicated that *ha33* was an early gene. Such an early expression start was not usually found for baculovirus structural proteins as structural genes usually contain baculovirus consensus late promoters [9]. However, *ha33* is probably not the only baculovirus gene which is expressed early in infection and encodes a structural virion protein, since the SeMNPV odv-e66, vp80capsid, vp39capsid, and p74 genes [14] also possess early promoter motifs. An alternative possibility is that the ha33 protein possesses different functions early and late in infection as Ijkel et al. [13] suggested for Se117. The ha33 protein was associated with the BV envelope but not with the ODVs, suggesting that the ha33 protein may either play a role in the assembly of BVs or relate to BV infection of cells, as the previous studies of baculovirus envelope proteins revealed that envelope proteins GP64 and Ld130 play important roles in attachment and budding from cells [11, 19, 20].

Homologous genes are present in all completely sequenced members of the lepidopteran nucleopolyhedroviruses as well as the granulovirus genus of baculoviruses. The genes are monophyletic, suggesting that they were acquired prior to the divergence of the two genera. Comparison also suggested that ha33 homologs are necessary specifically for lepidopteran NPVs and GVs as the gene is highly conserved in all lepidopteran baculoviruses but not present in non-lepidopteran baculoviruses, such as the completely sequenced dipteran baculovirus, *Culex nigripalpus* baculovirus, and the hymenopteran baculoviruses

Neodiprion sertifer nucleopolyhedrovirus and *Neodiprion lecontei* nucleopolyhedrovirus. Zanotto et al. (1993) showed that hymenopteran NsSNPV diverged from the lepidopteran baculovirus before the separation of the NPV from the GV and thus ha33 and its homologs were acquired prior to the divergence of NPVs and GVs but after the divergence of lepidopteran baculoviruses and hymenopteran NsSNPV. This result suggested that ha33 and its homologs possibly play an important role in the functions related to their lepidopteran hosts.

Ha33 contained a domain similar to nudix hydrolases. The name nudix is an acronym coined from the class of substrates hydrolyzed by these enzymes, predominantly **nu**cleotide **di**phosphates linked to some other moiety, **x**. The nudix hydrolases are widely distributed among species from bacteria to humans [15] and characterized by the signature sequence **$GX_5EX_7REUXEEXGU$** (where U is usually Ile, Leu, or Val) termed the nudix box [2, 27]. Substrates of nudix enzymes are either potentially toxic, deleterious compounds such as ADP-ribose (ADPR) and 8-oxo-GTP or important cell signaling molecules, regulators, and metabolic intermediates such as CoA, NADH, dATP, ApnA, and GDP-mannose [6]. The intra-cellular concentrations of both kinds of compounds require precise control and modulation during different parts of the cell cycle. These "housecleaning" activities appear essential for the well being of the organism [2]. In our study, the similar nudix sequence, **$GX_{10}EX_3$REFUEETG** (Fig. 1) were found in ha33 and its homologs in all sequenced lepidopteran NPV and GV genomes. To date, the function of the ha33 nudix domain has not been determined. In the early stages of the baculovirus life cycle, ha33 and its homologs possibly play a role as nudix hydrolases whose function is to cleanse the cell of potentially deleterious endogenous metabolites and to modulate the accumulation of intermediates of biochemical pathways [2], activities which may benefit baculovirus replication in the host cells. However, further studies are needed, particularly examination of virus mutants in which the gene has been deleted.

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