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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 TCID₅₀ units per cell) at 0, 3, 6, 12, 24, 48 and 72 hp.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 μ g 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

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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 BamHI site (underlined) and a downstream primer (5'-ACTCGAGTTAGTATTGCCGCTGCAT-3') with an XhoI site (underlined). The amplified fragment was inserted into pGEM-T easy vector (Promega, USA), retrieved by digestion with BamHI and XhoI, 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 \sim 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 TCID₅₀ 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

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						SI	NI.	ΤΙ	VI		
		* 20	· · · · · ·	40	*	60 -	80			-	
Ha33	:	MR-CSGLLIIMEP	DRAVLLCARRAY	NLSVANGN		HFLEKISIPRG	KWDSRDIFDYETA	VREFIEETG	TFFENA :	:	71
Hz33	:	MR-CSGLLIIMEP	DRAVLLCARRAY	NLSVANGN-		HFLEKISIPRG	KWD SRDIFDYETA	VREFIEETG	TFFENA :	:	71
Mc-B147	:	MR-CAGLFMIMEP	DKAVLLCARRSY	DSTVHYHD	ADQL	-NQVNFIEKISIPRGI	KRDGRDIFDYETA	VREFIEETG	TFFESA :	:	78
Mc-A148	:	MR-CAGI FULLMEP	DKAVLLCARRSY	DSTVHYHD	ADQL	-KQVNFIEKISIPRG	KRDGRDIFDYETA	VREFIEETG	TFFESA :		78
Se118	:	MHNSLFSFIMR-CAGEFORLEA	DKAVLLCARRSY	DSNVAYVDI	PAQL	-EHVNFLEKISIPRG	KRDGRDIFDYETA	VREFIEETG	TFFESA :	:	87
Ld46	:	MR-CAGLFLTVQP	dkavllcasrsy	CGHVR-HT:	SRSV	-DATNFLEKISIPRG	KRDGRDIFD <mark>YET</mark> A	VREFIEETG	CVEESA :		77
Ac38	:	MRNAAGE	DKAVLLCARRAY	RSANAPAAI	DM	NDTFLEKISIPRG	HRDCCDAKVYETA	VREFVEETG	RFFDSA :	:	76
Bm29	:	MRNAAGIFUTIEP	DRAVLLCARRAY	RSANAPAAI	DI	NDTFLEKISIPRG	HRDCCDAKVYETA	VREFVEETG	RFFDSA :	:	7€
Ro35	:	MRNAAGIFIIIEP	DKAVLLCARRAY	RSANAPAAI	NR	NDTFLEKISIPRG	HRDCCDAKVYETA	VREFVEETG	RFFDSA :	:	76
Cf22	:	MCKMRNSAGI FMIMEP	DKAVLLCARRAY	RGN-AVAS-		DTFLEKISIPRG	HRDCTDAKIYETA	VREFVEETG	RFFHSA :	:	75
Op22	:	MRNSAGLFMIMEP	DRAVLLCARRAY	HGG-AVS		DTFLEKISIPRG	hrdctdaki <mark>yeta</mark>	VREFVEETG	RFFHSA :	:	71
Cf-d22	:	MRNSAGE FOLLMEP	DKAVLLCARRSY	CGAASVNT		DTFLEKISIPRG	HRDCTDAKIYETA	VREFVEETG	RFFHSA :	:	73
Ep20	:	MCKMRNSAGIFMIMKP	DKAVLLCARRAY	RNAPMTTQ		DTFLEKISIPRG	HRDCTDAKIYETA	VREFVEETG	RFFHSA :	:	7€
Ah11	:	MR-SAGEFLIMEK	NRAILLNALKSY	KASDRFAM		TFAEKISIPRG	RRDGNDLFDYETA	VREFIEETG	TYFESA :		71
s135	:	MR-CSGLLLINDD	DEAILLHASKSY	APRRIYNG	GYDETVDEQEI	DEEDNFLEKISIPRG	KWD SRDIFDYETA	IREFIEETG	TVFDGA :	:	85
C1GV62	:	MVKRGKHAGILLITED	NKAVIL <mark>QANK</mark> SY	NESINKNL	KYN	-KHIPFVEKLSIPRG	KHDVGEK-DYETA	VREFIEETG	LVFDKV :	:	80
CpGV69	:	MVKRGKHAGLLLTED	NKAVILQANKSY	NENINKNLI	KYN	-KHIPFVEKLSIPRG	KHDVGEK-DYETA	VREFIEETG	LVFDKV :	:	80
AoGV59	:	MVKRGKHAGILLITEN	NKAVILOATKSY	NDNVNKNLI	KYN	-KHIPFVEKLSIPRG	KHDAGEK-DYETA	VREFIEETG	LVFDKI :	:	80
PoGV62	:	MVKHAGLLVITGD	NKAVILCANKSY	NDNVNRNLI	KYN	-KHIPFVEKLSIPRG	KHDAGEK-EYETA	IREFIEETG	LVFDRI :	:	77
AsGV61	:	MVKKGRHSGLLITND	dkaviloanksy	SERVNKNL	KYN	-KHIPFVEKLSIPRG	RQDVGEK-DYETA	VREFIEETG	LIFDKV :	:	80
XcGV79	:	MVKKGRHSCLLLTDD	DKAVILCANKSY	SEHVNKNLI	KYN	-KHIPFVEKLSIPRG	RODVGEL-DYETA	VREFIEETG	LIFDKI :	:	80
PxGV52	:	MVRKGRHSGLLLTDD	NHAVILQASKSY	SEHVNQNLI	KYN	KHIPFVEKLSIPRG	RODVGET-DYETA	VREFIEETG	LIFDKI :	:	80
				73		175					
	3	71 T2	67			¥2			S4		
	-	<u>100</u> · · ·	120 5		140	<u> </u>		180	*	-	
Ha33	:	YVYRSPFLLH@QDHGVVYRYTI	WGIHKC-PHRT	WSRKPNIN	CWRDQR	-NNRDSNEWQFNuQVI	RRH-NHEIIPRNIY	IWPHKDWFQ	YMRESC	•	155
Hz33	:	YVARSPELLHWODHGVVVRATI	WGIHKC-PHRT	WSRRPNTY	CWREQR	NNRDSNEWQFNDQVI	RRH-NHEIPRNIY	IVPHKDMFQ	YMRESC :	•	155
Mc-B147	:	WWRVEFVIQUNDAGVTWRMAI	INGV/QC-LIPRN	WSREDNTY	CWRIDN	CDKPNDWK INMETI	RRH-NNEHPRNHY	III PII QDMF Q	YMNERC	•	164
Mc-A148	:	WVYRVEEVLOWNDAGVTYRYAL	rwgwwQ G -Lirrn	IVSREPNTY	OVKENN	CDKPNDYK NETI	RRH-NNEHPRNHY	TUBHQDMFQ	YMNERC	•	164
Sell8	÷	WVERVEFVILQWNDAGVTYNMAL	INGANKG-TUAH	IVSREENTY	CWRIDN	-CORPNDERFNDEVI	RRY-NNELPRYLY	THATODAFO	YMNERC		173
Ld46	:	YVARAPEVIIHMQDAGVTYTYSI	NUGVTVGAAI KO	WAREENTE	CWRIIQRGRA	AAAADQNIMRUNIEKI	RRF-NNBHARRITY	III SI DRAFO	YMNERC	•	165
AC38	•	FIXELEFTICMEDDGATASATI	YWGVVRG-NITIN	WNARPNTY.	LAKT P5	GTFGNDWR MUKPI	REF-NCELARSHA	OP NKYPN	YMNDRC	•	163
Bm29	:	FLARFEFTLOWRDDGVTMRMLL	INGVIRG-NITID	WNARPNTY:	PURDLP	GTFGNDWRIMMKPI	RRF-NCELTRSLA	PUNKERN	YMINDRO		163
R035	:	FLAKTEFLUGWKDDGALAKATT	YVGVVRG-NITIN	WNARPNTY.	PORLEP	GTFGNDWRUMERPI	RRF-NCELARSHA	I VPII NKYPN	YMINDRO		163
0122	:	YLYKFEFTLHWTDEGVTYKYSL	IVGVVRG-AHAD	VKYRPNTY.	PWKELP	GALGNDWR VERPI	ORF-NCELSRSHA	IVENDRAFE	YNNSKC		162
0022	•	YIYKFPFTLHWTDEGVTYKYSI	YVGVVRG-AMAD	VEF RENTY.	TOKEEP	GAN-NDWR VIRPI	RRF-NCELSRSHT	INPHINOMED	YMTSKC :		157
CI-022	•	FIXEFEETIHWTDRGVTXEXSL	rvgivrgCAD	WEFEENTY	WAKTTE	GAFGYDMR VIRPI	RRF-NCELARSVT	L PHOYLE	YMT SRC		102
Ep20	•	TIRFEFTLHWTDDGVTIRISI	NGVUNG- SHAN	VAP RENTT.	TVKLLP	GOLUGNDAR VERP	RE-NCELVRSVT	TOPHNOTED	TIMT NICE		163
ANII	•	AAANABAAT DOWNDDGAAAKAAT	TIGINKG-INUT	TPOSPNIP	OVREKS	- SSYKSNDAN YUTTI	RRQ-NNELERNLY	I STINEYYO	YMHERC		132
5135	:	TYTERPEPERMTDRSVNTTMI	TVALING-NIKE	TRRENTRO	VKLLK CPI	T NOCMERSED DOUD	CRIANNEL SRADC	TAM MY AT S	TWREEL :		171
CIGV62	:	PUENEDEULEMODNCKMYRYUM		TREDDIGU	MIKIKGRI	F-CDEVENEUDILE	OF DELETION OF DELETION	DANI CRATS	UMP GP C		170
CpGV65	:	CURCEDEMINICONCURCEMINI	IVAP MG_ m VV	TVVVDNSV	THETRADADTCOL	A OTVICEN DULL	WERTING WERDS	N N SVST	MIND O		174
Rogue2	:	CURADDRULEMODNERUMAN	VUAR T.C. R. VV	TVDVDNGVI	TWIN V	DNUNEWEWEWEUDET.D	WED GUET UDD TE	TAN DONTO	MIT MILE		145
A a GUG1	:	STYNDET TEMODE STYNDE	WARDOG - GUNG	TDVVDNOVI		VOVN-VOVENDIOV	DYCMMET VENTE		MARINE OF A		167
Xacu79	:	STIRDEFIDEMODD PYMELYSM	NAFING-SHNS	DERKENST		D ST. NIMENDIODI SD	PHUMPLICATION	CONTRACTOR	VMP MP C		169
PxGV52	:	ATYKEPEEDEMODDCKTEBYTV	WAFING-TINS	TSKBPNSY		CKLE-NSWEWDWCK	KHRSKETMBRIE	TUSIKKYUT	YMENBO		167
	8				-					8.0	
		S4 Y	3								
		<u> </u>	220	*	240	* 2	* 002	28	0		
Ha33	:	LVTYDSSNYLDEFDFVEQVEID	ID SGQLNK	-FFTLSLR	NVPIRTPAIII	SEKRVQFHGADKTA	VLRVQQRHGQRNM	QRQY	;	: 1	238
Hz33	:	LVTYDSSNYLDFFDFVEQVKID	TD SGQLNK	FFTLSLR	NVPIRTPAIII	SEKRVQFHGADKTA	VLRVQQRHGQRNM	QRQY	;	: 1	238
Mc-B147	:	LVTYDSSNYLEEFEFVKSVRAK	PDQKNLRR	-FFLLSLK-	LESFDFFH	KWMRRAPRI	ELMLTTNTNLKRI	VNAV	;	: 1	234
Mc-A148	:	LVTYDSSNYLEFFEFVKSVKAK	FDQRNLRR	-FFLLSLK-	LESFDFFH	KWMSRGPRI	PLMLTPNKI	VNVV	;	:)	230
Se118	:	LTHYDSSNYLKVFEFVKSVKSK	FDRHNLIN	-FFYLTLK	LDTFNFYH	TWDKRTQQQQQQRRR(QQQQQKQPSSPQY	YTGVYKGFK	ENHQCV :	: }	261
Ld46	:	LVHYDSSNYLDEFIFVRAVKRE	PDSGETLN	-FFCLSLQ-	LETLAAGLI	DERKRDK	WRPPSSARPNY	VAATRQALR	EIGTIV :	: 1	247
Ac38	:	LITYDYSNYIEFFDFVRSVKAR	FDNRQLQD	-FFYATLK-	KIDN-DAP	QKLHALRRV			;	: 3	216
Bm29	:	LITYDYSNYIEFFSFVRSIEKR	FDNRQLQD	-FFYATLK-	KIDNNDAP(QKLHALRRV			;	: :	217
Ro35	:	LITYDYSNYIEFFDFVRSVKAR	TD SRQLQD	-FEYATLK-	KIDY-DAPO	QKLHSLRRV			;	: 1	216
Cf22	:	LNTYASSNYSEFFDFVRQVERL	FDIKQLHD	-FEHASLE	RV				;	:	201
Op22	:	LNTYASSNYGEFFDFVRQVKRL	DNKQLHD	-FFHASLQ-	RVDPNDAL	ACPGPQR			;	: 1	205
Cf-d22	:	LNAYASSNYTEFFNFVRQVREL	DNKQLHD	-FFYASIK	RVEVA				;	: 1	201
Ep20	:	LN#YASSNYTEREKFVIQVRQL	DINEQLED	-FEYASIK-	RDEPHDVK:	SSRL			;	:	212
Ah11	:	LVHYRSSNYVQFFEYTEIIRNK	DCCOLKD	-FELLRIH	FSSPSPII:	SPCRRKK	-ISSAIKTELRNI	ITRA	;	:	227
s135	:	LTRYEASNYLDEFGFVETVRAD	MDKNPDDDP	RFFFFIQLQ-	WKKRTTIVA	4			;	•	220
CIGV62	:	D SHYKYSNYDVEFNYTYMVREL	MAETHFE-	TERQLDUM	WYVESEKYNLI	GCY			;	•	221
CpGV69	•	LSHWKYSWYEVSEDWITYTWRRL	AGTYD-	ARE OF DRW	WYVESERYNLI	LCY			;	•	220
AoGV59	:	LSHYKYSNYLLEFDYVYSVQKM	SNFELD-	NEEFIDEQU	NCVESEEFDV	KCCL			;		225
POGV62	•	ICHYKYSWYRH: FNYHKNVEHV	KSGDSS-	REFEIDEM	NCVDSEKCSQI	/TX			;		215
ASGV61	:	TDEAVYSIN SPENDOR VYKORRL	LSNDLI-	MELVILLY	WINDSETVSLI	ALLERT					22E
ACGV/9	:	HDUVKVSNYDVREMEUROUSOW	PMN	DESTITUT	INFSO	VE TII A					240
L X0102	•	DE ENTRATION DIVISION DE CARON		- Carlow Contraction of the	**************************************				Non-Weiter Certrand		-01

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

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 orf22	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

 Table 1. Homologous ORFs of ha33 among baculovirus genomes

M 0 3 6 12 24 48 72 h p.i.



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 GX5EX7REUXEEXGU (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, $GX_{10}EX_3REFUEETG$ (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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References

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402
- 2. Bessman MJ, Frick DN, O'Handley SF (1996) The MutT proteins or "nudix" hydrolases, a family of versatile, widely distributed, "housecleaning" enzymes. J Biol Chem 271: 25059–25062
- Chen X, Ijkel WF, Tarchini R, Sun X, Sandbrink H, Wang H, Peters S, Zuidema D, Lankhorst RK, Vlak JM, Hu Z (2001) The sequence of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus genome. J Gen Virol 82: 241–257

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- 4. Cherbas L, Cherbas P (1993) the arthropod initiator: the capsite consensus plays an important role in transcription. Insect Biochem Mol Biol 23: 81–90
- 5. Cunningham JT (1998) North America. In: Fujita-Hunter FR, Entwistle Ph F, Evans HF, Crook NE (eds) Insect viruses and pest management. John Wiley, New York, pp 313–331
- 6. Dunn CA, O'Handley SF, Frick DN, Bessman MJ (1999) Studies on the ADP-ribose pyrophosphatase subfamily of the nudix hydrolases and tentative identification of trgB, a gene associated with tellurite resistance. J Biol Chem 274: 32318–32324
- Durantel D, Croizier G, Ravallec M, Lopez-Ferber M (1998a) Temporal expression of the AcMNPV lef-4 gene and subcellular localization of the protein. Virology 241: 276–284
- 8. Durantel D, Croizier L, Ayres MD, Croizier G, Possee RD, Lopez-Ferber M (1998) The pnk/pnl gene (ORF86) of *Autographa californica* nucleopolyhedrovirus is a nonessential, immediate early gene. J Gen Virol 79: 629–637
- 9. Funk CJ, Braunagel SC, Rohrmann GF (1997) Baculovirus structure. In: Miller LK (ed) The baculoviruses. Plenum Press, New York, pp 7–32
- 10. Guarino LA, Smith G, Dong W (1995) Ubiquitin is attached to membranes of baculovirus particles by a novel type of phospholipid anchor. Cell 80: 301–309
- Hefferon KL, Oomens AG, Monsma SA, Finnerty CM, Blissard GW (1999) Host cell receptor binding by baculovirus GP64 and kinetics of virion entry. Virology 258: 455–468
- 12. Ignoffo CM (1973) Development of a viral insecticide: concept to commercialization. Exp Parasitol 33: 380-406
- Ijkel WF, Lebbink RJ, Op den Brouw ML, Goldbach RW, Vlak JM, Zuidema D (2001) Identification of a novel occlusion derived virus-specific protein in *Spodoptera exigua* multicapsid nucleopolyhedrosis virus. Virology 284: 170–181
- 14. Ijkel WF, van Strien EA, Heldens JGM, Broer R, Zuidema D, Goldbach RW, Vlak JM (1999) Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. J Gen Virol 80: 3289–3304
- Kang LW, Gabelli SB, Cunningham JE, O'Handley SF, Amzel LM (2003) Structure and Mechanism of MT-ADPRase, a Nudix Hydrolase from Mycobacterium tuberculosis. Structure 11: 1015–1023
- Kang W, Suzuki M, Zemskov E, Okano K, Maeda S (1999) Characterization of baculovirus repeated open reading frames (bro) in *Bombyx mori* nucleopolyhedrovirus. J Virol 73: 10339–10345
- 17. McIntosh AH, Ignoffo CM (1983) Characterization of five cell lines established from species of Heliothis. Appl Entomol Zool 18: 262–269
- O'Reilly DR, Miller LK, Luckow VA (1992) Baculovirus expression vectors: a laboratory manual. WH Freeman & Co., New York
- 19. Oomens AG, Blissard GW (1999) Requirement for GP64 to drive efficient budding of *Autographa californica* multicapsid nucleopolyhedrovirus. Virology 254: 297–314
- 20. Pearson MN, Groten C, Rohrmann GF (2000) Identification of the *Lymantria dispar* nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the *Baculoviridae*. J Virol 74: 6126–6131
- 21. Pearson MN, Rohrmann GF (1997) Splicing is required for transactivation by the immediate-early gene 1 of the *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus. Virology 235: 153-165
- 22. Pullen SS, Friesen PD (1995) Early transcription of the ie-1 transregulator gene of *Autographa californica* nuclear polyhedrosis virus is regulated by DNA sequences within its 59 noncoding leader region. J Virol 67: 5776–5785
- 23. Rohrmann GF (1999) Nuclear polyhedrosis viruses. In: Webster RG, Granoff A (eds) Encyclopedia of virology, 2nd edn. Academic Press, London, UK

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- Sun XL, Zhang GY, Zhang ZX, Hu ZH, Vlak JM, Arif BM (1998) In vivo cloning of *Helicoverpa armigera* single nucleocapsid nuclear polyhedrosis virus genotypes. Virol Sin 13: 83–88
- 25. Theilmann DA, Stewart S (1993) Analysis of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus trans-activators IE1 and IE2 using monoclonal antibodies. J Gen Virol 74: 1819–1826
- 26. Winstanley D, O'Reilly DR (1999) Granuloviruses. In: Webster RG, Granoff A (eds) Encyclopedia of virology, 2nd edn. Academic Press, London, UK
- 27. Xu WL, Dunn CA, Bessman MJ (2000) Cloning and characterization of the NADH pyrophosphatases from *Caenorhabditis elegans* and *Saccharomyces cerevisiae*, members of a nudix hydrolase subfamily. Biochem Biophys Res Commun 273: 753–758
- 28. Zanotto PM, Kessing BD, Maruniak JE (1993) Phylogenetic Interrelationships among baculoviruses: Evolutionary Rates and Host Associations. J Invertebr Pathol 62, 147–164
- 29. Zhang CX, Ma XC, Guo ZJ (2005) Comparison of the complete genome sequences between C1 and G4 isolates of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus. Virology 333: 190–199

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