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Open reading frame 132 of *Heliocoverpa armigera nucleopolyhedrovirus* encodes a functional *per os* infectivity factor (PIF-2)

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Open reading frame 132 (*Ha132*) of *Helicoverpa armigera nucleopolyhedrovirus* (HearNPV) is a homologue of *per os* infectivity factor 2 (*pif-2*) of *Spodoptera exigua multiple nucleopolyhedrovirus*. Sequence analysis indicated that Ha132 encoded a protein of 383 aa with a predicted molecular mass of 44·5 kDa. Alignment of HA132 and its baculovirus homologues revealed that HA132 was highly conserved among baculoviruses, with 14 absolutely conserved cysteine residues. RT-PCR indicated that *Ha132* was first transcribed at 24 h post-infection. Western blot analysis showed that a 43 kDa band was detectable in HearNPV-infected HzAM1 cells from 36 h post-infection. Western blots also indicated that HA132 was a component of the occlusion-derived virus, but not of budded virus. Deletion of *Ha132* from HearNPV abolished *per os* infectivity, but had no effect on the infectivity of the budded virus phenotype.

INTRODUCTION

Baculoviruses are large, double-stranded DNA viruses pathogenic to a variety of insect species. Naturally occurring and modified baculoviruses are potentially viable as environmentally benign biological-control agents against economically important forest and agricultural insect pests (Wood & Granados, 1991). The baculovirus *Helicoverpa armigera nucleopolyhedrovirus* [HearNPV, also called *Helicoverpa armigera* single nucleocapsid polyhedrovirus (HaSNPV)] is a naturally occurring pathogen of cotton bollworm and was first isolated from diseased larvae in the Hubei province of China. It has been developed as a successful commercial pesticide for the control of cotton bollworm in China (Zhang, 1994).

To understand the genetic properties of HearNPV and further improve its effectiveness, the complete nucleotide sequence and genetic organization of HearNPV have been elucidated (Chen *et al.*, 2001). The virus was modified genetically by deleting the ecdysteroid UDP-glucosyltransferase (*egt*) gene from its genome or by inserting an insect-selective scorpion toxin gene that has been shown to improve the viral insecticidal property (Chen *et al.*, 2000; Sun *et al.*, 2004). We are now focusing on studying other genes that may contribute to the insecticidal property of the virus. *Per os* infectivity factors are among the genes of interest, as they may serve as targets for future genetic engineering to enhance the oral infectivity of baculoviruses.

Recently, *Se35* has been identified as encoding a *per os* infectivity factor (PIF-2) of *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV) (Pijlman *et al.*, 2003). It has been known that the PIF-2 homologue (*Ac22*) in *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) is a structural component of the occlusion-derived virus (ODV) (Braunagel *et al.*, 2003). Although the gene is conserved in baculoviruses, its functionality in viruses other than SeMNPV has not yet been elucidated. NPVs are designated single (S) or multiple (M) NPVs based on whether the ODV that initiates primary midgut infections contains single or multiple nucleocapsids. Washburn *et al.* (2003) revealed that, in orally inoculated larvae of *Heliothis virescens*, *Helicoverpa zea single nucleopolyhedrovirus* (HzSNPV) initiated primary infections quicker and in greater numbers than AcMNPV, implying differences between SNPVs and MNPVs in primary midgut infection.

In this report, we characterized open reading frame (ORF) 132 of HearNPV (*Ha132*), a homologue of *Se35*, and studied its function by deleting it from the virus. RT-PCR was

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performed to detect transcription and Western blot analysis was used to identify whether HA132 was a component of the virus structure. Recombinant viruses HearNPVΔ132 with deletion of *Ha132* were constructed from wild-type (wt) HearNPV. The infectivity of budded virus (BV) and ODV was examined *in vivo*. The data presented in this manuscript ascertain that *Ha132* is functional in HearNPV.

METHODS

Computer-assisted analysis. A similarity search of the *Ha132* gene and its deduced amino acid sequences was performed against updated GenBank/EMBL, SWISS-PROT and PIR databases by using BLASTP, FASTA and PSI-BLAST programs (Pearson, 1990; Altschul *et al.*, 1997). HA132 was analysed by using software of the ExPASy server (Appel *et al.*, 1994) to predict domains and motifs (Reinhardt & Hubbard, 1998). Multiple sequence alignments were performed with CLUSTAL_X (Thompson *et al.*, 1997). Alignment editing was performed with GeneDoc software (Nicholas *et al.*, 1997). The following motifs were searched as potential transcription start sites of *Ha132*: early promoter motifs TATAA, ATCA(G/T)T and CGTGC (Blissard & Rorhmann, 1990), baculovirus late transcription start site DTAAG (Blissard & Rorhmann, 1989) and downstream activating elements (A/T)CACNG (Friesen, 1997).

Insect cells and virus. The *Helicoverpa zea* cell line HzAM1 (McIntosh & Ignoffo, 1983) was maintained at 28 °C in Grace's medium supplemented with 10 % fetal bovine serum. HearNPV strain G4, the genome of which has been sequenced entirely (GenBank accession no. AF271059; Chen *et al.*, 2001), was deemed as wt and propagated in HzAM1 cells.

RNA isolation and RT-PCR. HzAM1 cells were infected by wt HearNPV at an m.o.i. of 5 and total RNA was isolated with TRIzol (Gibco-BRL) at 0, 4, 8, 16, 24, 48 and 72 h post-infection (p.i.). RT-PCR was performed with 1 μg total RNA as template per time point. First-strand cDNA synthesis was performed by using AMV (avian myeloblastosis virus) reverse transcriptase (Promega) and a 15mer oligo-dT primer (Takara) according to the manufacturer's instructions. The cDNA mixtures were amplified with PCR by using the 132down primer (5′-GGGAAGCTT*TTACGACGGCAAATCCCTACG*-3′) (the *Hin*dIII site is underlined and the italic sequence is complementary to nt 127750–127770 in the HearNPV G4 genome) and a primer internal to *Ha132* (132in) containing an *Eco*RI site (underlined) (5′-GAATTC*AAAATATGAGTCAGG*-3′) (the italic sequence corresponds to nt 127122–127137 in the HearNPV G4 genome).

Western blot analysis. Monolayers of HzAM1 cells were infected by wt or recombinant HearNPV at an m.o.i. of 5. Infected cells were harvested at 0, 8, 16, 24, 36, 48 and 72 h p.i. BV and ODV were purified according to IJkel *et al.* (2001). Samples of total cell proteins, BV and ODV were separated by SDS-PAGE and transferred onto a Hybond-N membrane (Amersham Biosciences) for Western blotting. The primary antibody was a polyclonal, HA132-specific antiserum generated from rabbits immunized with purified HA132–glutathione *S*-transferase expressed in *Escherichia coli*. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Gibco-BRL) was used as the secondary antibody. The signal was detected by using a BICP/NBT kit (Sino-America).

Deletion of *Ha132* from HearNPV. To construct an *Ha132* deletion mutant, a transfer vector was constructed as follows: the upstream flanking sequence of *Ha132* was amplified by PCR with the primers P1 containing a *Hin*dIII site (underlined), 5′-GGGAAGCTT*TGTTGCGGGGTTACGAAGAGC*-3′ (the italic sequence corresponds to nt 125131–125151 in the HearNPV G4 genome) and P2 with a *Pst*I site (underlined), 5′-CCCCTGCAG*CAATAGCAGCCAGATCAACAT*-3′ (the italic sequence is complementary to nt 126599–126621 in the HearNPV G4 genome). The downstream flanking sequence of *Ha132* was obtained by PCR with the primers P3 containing a *Kpn*I site (underlined), 5′-GGGGGTACC*TTCGTAGGGATTTGCCGTCGT*-3′ (the italic sequence corresponds to nt 127748–127768 in the HearNPV G4 genome) and P4 containing an *Eco*RI site (underlined): 5′-GGGGAATTC*AAACGAAACATTGGATTGAACTT*-3′ (the italic sequence is complementary to nt 129309–129331 in the HearNPV G4 genome). The two PCR products were first cloned into pUC19 to generate p132LR. The *lacZ* gene was cloned into p132LR to generate p132LR-LacZ. Co-transfection of wt HearNPV and p132LR-LacZ in HzAM1 was performed as described by King & Possee (1992). Recombinant plaques were identified by blue colour and polyhedron formation. The deletion mutant HearNPVΔ132 was purified by three rounds of plaque purification and identified by restriction-enzyme analysis.

***In vivo* infectivity assay.** The infectivity of BV was examined by injecting 10 μl supernatant with a titre of 10^5 $TCID_{50}$ ml^{-1} into the third-instar larvae of *H. armigera*. Grace's medium was used as a negative control. For the oral-infectivity assay, polyhedra of wt HearNPV and HearNPVΔ132 were purified from diseased larvae as described by Sun *et al.* (1998). The infectivity was assayed in neonate and third-instar *H. armigera* larvae by diet contamination, using 10^6 occlusion bodies (OBs) per larva. Infected larvae were reared individually in 24-well plates and monitored daily until all larvae had either pupated or died.

RESULTS

Sequence analysis of HearNPV ORF132 and its homologues

The *Ha132* gene is located in the *Hin*dIII–D fragment of the HearNPV genome. A late baculoviral transcript motif TTAAG was found 13 nt upstream of the putative translational start site, suggesting that *Ha132* may be transcribed late in infection. A polyadenylation signal, AATAAA, was identified at nt 249–254 downstream of the TAA stop codon.

Appropriate searches of protein databases showed that the putative HA132 protein and homologues were highly conserved among all baculoviruses whose genomes have been sequenced so far. Alignment of HA132 homologues from SeMNPV (a group II MNPV), AcMNPV (a group I MNPV), *Xestia c-nigrum granulovirus* (XcGV), *Neodiprion sertifer nucleopolyhedrovirus* (NeseNPV; a hymenopteran NPV) and *Culex nigripalpus nucleopolyhedrovirus* (CuniNPV; a dipteran NPV) revealed that the predicted amino acid sequence of HA132 shared identity ranging from 66 % with SeMNPV ORF35 to 44 % with NeseNPV ORF55 (Fig. 1). As expected, HA132 is related most closely to ORF97 of HzSNPV with 100 % identity, as HzSNPV is a substrain of HearNPV (Chen *et al.*, 2002). The similarity was distributed throughout the sequence and there were several regions where the sequences were more highly conserved. One striking phenomenon was that all 14 cysteine residues of HA132 were completely conserved among this group of proteins (Fig. 1). This indicates that the protein can form multiple disulfide bonds. The N terminus of HA132 was found to be highly hydrophobic, with 13 of the first 19 aa consisting of Leu, Ile and Val. This

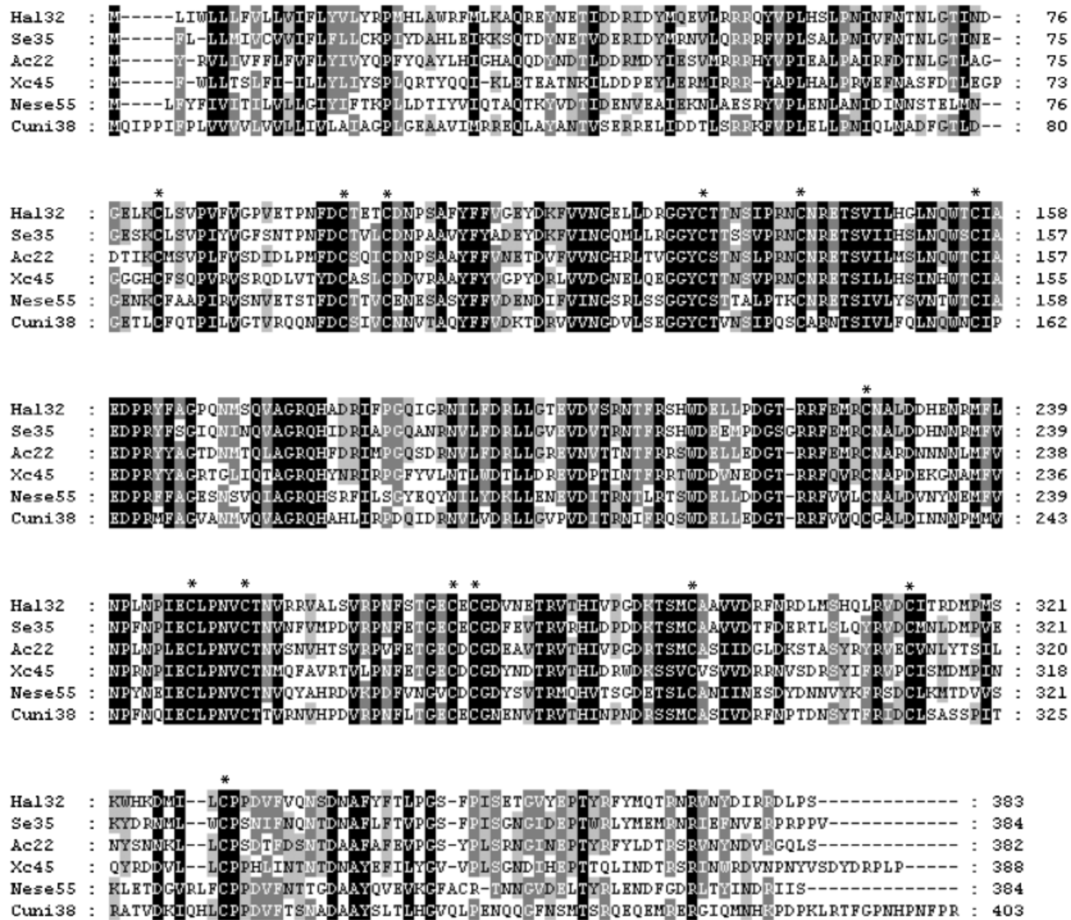

Fig. 1. Alignment of HearNPV ORF132 and its homologues. The 14 absolutely conserved cysteines are marked with asterisks. GenBank accession numbers are: AAG53875 for HearNPV ORF132 (Ha132), AAF33565 for Se35, AAA66652 for ORF22 of AcMNPV (Ac22), AAF05159.1 for ORF45 of XcGV (Xc45), AAQ96432 for ORF55 of NeseNPV (Nese55), AAK94116 for ORF38 of CuniNPV (Cuni38). GeneDoc software was used for similarity shading.

hydrophobic N-terminal region was conserved in all of the HA132 homologues (Fig. 1). This region was also predicted as a transmembrane domain by TMPRED (Hofmann *et al.*, 1993).

Transcription analysis of the *Ha132* gene

RT-PCR was performed to study the transcription of *Ha132*. One primer internal to *Ha132* (132in) and primer 132down were used to amplify a fragment of 650 bp. Consistently, an *Ha132*-specific sequence was amplified at 24 h p.i. and increased at 48 and 72 h p.i. (Fig. 2). The data demonstrate that Ha132 is a late gene, as the replication of viral DNA in HzAM1 cells started at 7 h p.i. (W. T. Dai, personal communication). Sequence analysis of the upstream and downstream regions indicated that there was a baculovirus late promoter motif TTAAG 13 nt upstream of the start codon ATG. As it has been shown previously by primer extension that the *p6.9* gene of HearNPV used ATAAG as its late promoter (Wang *et al.*, 2001), this TAAG motif upstream of *Ha132* is likely to be functional.

Immunodetection of HA132 in the infected cells and in BV and ODV

Western blot analysis of extracts of HearNPV-infected HzAM1 cells revealed a specific protein band of 43 kDa

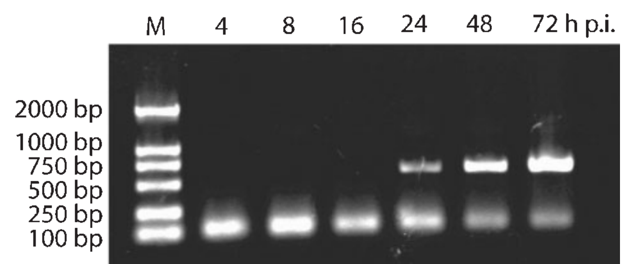

Fig. 2. Transcription analysis of the *Ha132* gene. RT-PCR analysis was performed on total RNA extracted from HearNPV-infected HzAM1 cells at different time points p.i. by using primers 132down and 132in.

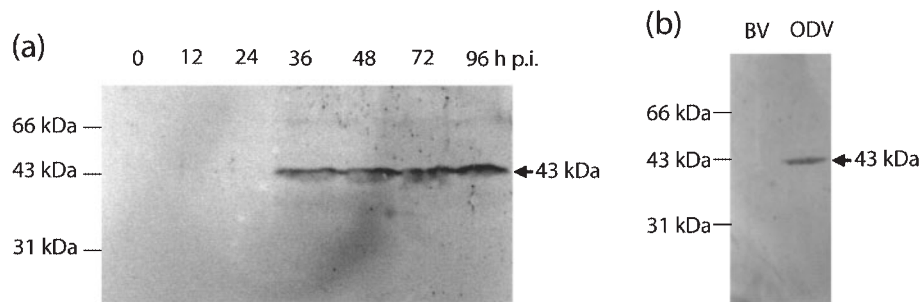

Fig. 3. Western blot analysis of HA132. (a) Western blot analysis of HA132 in HearNPV-infected HzAM1 cells from 0 to 96 h p.i. Lane numbers correspond to time p.i. (b) Western blot analysis of HA132 in the purified BV and ODV. Size standards are indicated on the left; the single 43 kDa immunoreactive protein is indicated on the right.

from 36 to 72 h p.i. (Fig. 3a). The size of the 43 kDa protein is close to the predicted 44·5 kDa size of the putative Ha132 translational product, suggesting no major post-translational modifications.

To investigate whether HA132 is a structural component of HearNPV, Western blot analysis of BV and ODV was conducted. Results showed that HA132 was detected in preparations of ODV, but not in BV (Fig. 3b), suggesting that HA132 is a structural component of ODV.

Construction of HearNPVΔ132

HearNPVΔ132, an *Ha132* deletion mutant, was constructed as described in Methods. *Hin*dIII restriction-digestion profiles of HearNPVΔ132 were compared with those of wt HearNPV (Fig. 4a). The data showed that the *Hin*dIII–D fragment (12·9 kb) from the wt HearNPV genome had disappeared, with a concomitant appearance of two fragments of 8·1 and 7·2 kb in the HearNPVΔ132 profile. The digestion proved that the correct deletion mutant was produced.

Western blot analysis was performed to confirm further that total expression of *Ha132* was eliminated in HzAM1 cells infected with HearNPVΔ132 (Fig. 4b). HA132 was detectable in wt HearNPV-infected cells at 72 h p.i., but was undetectable in HearNPVΔ132-infected cells at different times post-infection. This result confirmed that *Ha132* was deleted in HearNPVΔ132.

HearNPVΔ132 lost its oral infectivity, but retained its BV infectivity

To study the infectivity of BV and ODV of HearNPVΔ132, three bioassay experiments were carried out (summarized in Table 1). Firstly, BVs of wt HearNPV and HearNPVΔ132 were injected into the haemolymph of third-instar *H. armigera* larvae. Mortalities due to HearNPVΔ132 and wt viruses were about 92 and 94 %, respectively (Table 1), whereas larvae injected with Grace's medium (negative

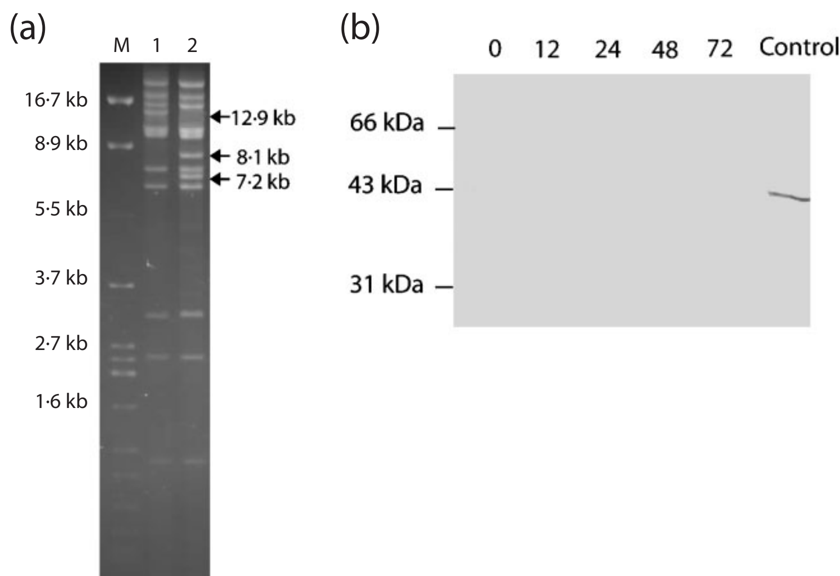

Fig. 4. Analysis of *Ha132* deletion recombinant HearNPVΔ132. (a) Restriction-enzyme digestion analysis of wt HearNPV (1) and HearNPVΔ132 (2) by *Hin*dIII. The different bands are indicated by arrows and their sizes are shown. (b) Western blot analysis of total cell proteins from HearNPVΔ132-infected cells using HA132 polyclonal antisera. The numbers above lanes represent different times post-infection (h p.i.). wt HearNPV-infected cells at 72 h p.i. were used as a positive control for Western blot.

Table 1. Comparison of infectivity of wt HearNPV and HearNPVΔ132

Virus	BV/ODV	Viral dose per larva	Inoculation method	Larval stage*	Total no. larvae	No. dead larvae	Mortality (%)
wt HearNPV	BV	10 μl	Injection	L3	260	245	94
HearNPVΔ132	BV	10 μl	Injection	L3	256	235	92
wt HearNPV	ODV	10^6 OBs	Oral	Neonates	228	179	78
HearNPVΔ132	ODV	10^6 OBs	Oral	Neonates	225	2†	–
wt HearNPV	ODV	10^6 OBs	Oral	L3	140	134	96
HearNPVΔ132	ODV	10^6 OBs	Oral	L3	283	1†	–

*L3, Third instar.

†Neither evidence of virus infection nor polyhedra were found in the dead larvae.

control) survived (data not shown). OBs of wt HearNPV and HearNPVΔ132 were fed to neonate or third-instar *H. armigera* larvae by diet contamination. The results showed that HearNPVΔ132 was not infectious to *H. armigera* larvae by oral ingestion (Table 1). Therefore, the deletion of *Ha132* resulted in the complete lost of *per os* infectivity, but did not affect BV infectivity.

DISCUSSION

In this paper, we reported the sequence analysis, transcription, protein identification and function of *Ha132*, a homologue of *per os* infection factor 2, in HearNPV.

The HA132 antiserum identified a 43 kDa polypeptide from HearNPV-infected cell lysates (Fig. 3a). HA132 was first detected at 36 h and continued to be present at 96 h post-infection. This type of temporal expression is consistent with the finding that HA132 is an ODV structural protein. The N terminus of HA132 contains a hydrophobic region that is conserved in its homologues. It has been reported that some ODV proteins contain an N-terminal hydrophobic structure, such as OpMNPV P91 (Russell & Rohrmann, 1997), AcMNPV ODV-E66 and ODV-E25 (Hong *et al.*, 1997). PIF-1 and SE35 also contain a similar structure (Kikhno *et al.*, 2002; Pijlman *et al.*, 2003). The hydrophobic domain of PIF-1, PIF-2, ODV-E66 and ODV-E25 is also rich in isoleucine, leucine and valine (Figs 1, 5; Hong *et al.*, 1997; Kikhno *et al.*, 2002; Pijlman *et al.*, 2003). It has been reported that the N-terminal hydrophobic domains of ODV-E66 and ODV-E25 are uncleaved in the ODV envelope and they were sufficient to direct reporter proteins to the nuclear envelope, intranuclear microvesicles and the ODV envelope within baculovirus-infected cells (Hong *et al.*, 1997). This sequence, called the sorting motif (SM), contains two features, a hydrophobic sequence and associated charged amino acids oriented on the cytoplasmic/nucleoplasmic face. It is proposed that, once inserted into the endoplasmic reticulum (ER), the SM protein interacts with the viral proteins FP25K and/or E26 during trafficking to the nuclear envelope (Braunagel *et al.*, 1999, 2004; Rosas-Acosta *et al.*, 2001). From the comparison (Fig. 5) of N-terminal sequences of PIF-1, PIF-2, PIF-3, ODV-E66 and ODV-E25, all of the PIFs possess the same characteristics of the SM as ODV-E25 and ODV-E66, suggesting that these PIFs are transported to the inner nuclear membrane and intranuclear vesicles by the same pathway as ODV-E25/ODV-E66. Whether the N-terminal hydrophobic domains of PIFs function in a manner similar to those of ODV-E66 and ODV-E25 needs to be investigated further.

So far, four conserved genes, *p74*, *pif-1*, *pif-2* and *pif-3*, have been identified to be essential factors for *per os* infectivity of baculoviruses. P74 has been described previously in AcMNPV as a structural ODV protein required for infectivity of polyhedra (Kuzio *et al.*, 1989; Faulkner *et al.*, 1997). Deletion or disruption of the AcMNPV *p74* gene results in the complete elimination of the *per os* infectivity of OBs, while virions purified from mutant OBs were infectious when injected into the haemocoel of *Trichoplusia ni* and *Heliothis virescens* larvae (Faulkner *et al.*, 1997; Haas-Stapleton *et al.*, 2004). Competing assays indicated that P74 might function as an ODV attachment protein that binds to a specific receptor on primary target cells within the midgut

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ODV-E25 Ac94   MWGIVLLIVLLILFYLYWTNALNFNSLTESSPSLG
ODV-E66 Ac46   M-SIVLIIVIVVIFLICFLYLSNSNNKNDANKNNA
PIF-1 Ac119    M-HFAIILLFLLVIIAIVYTYVDLIDVHHEEVRYP
PIF-1Spli7     MYKILLIVLFLVVVLILVRYVGQLYRVEDEKADRD
PIF-2 Ac22     MYR-VLIVFFLFVFLYIVYQPFYQAYLHIGHAQQD
PIF-2 Ha132    MLIWLLLFVLLVIFLYVLYRPMHLAWRFMLKAQRE
PIF-2 Se35     MF-LLLMIVCVVIFLFLLCKPIYDAHLEIKKSQTD
PIF-3 Ac115    MLNFWQILI-LLVIILIVYMYTFKFVQKFILQDAY
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Fig. 5. Comparison of N-terminal sequences of PIF-1, PIF-2, PIF-3, ODV-E25 and ODV-E66. GenBank accession numbers of these amino acid sequences are: AAA66724 for ODV-E25, AAA66676 for ODV-E66, AAA66749 for PIF-1 Ac119, AAM93424.1 for PIF-1 Spli7, AAA66652 for PIF-2 Ac22, AAF33565 for PIF-2 Se35, AAG53875 for PIF-2 Ha132 and AAA66745 for PIF-3 Ac115. Shaded amino acids indicate the strong hydrophobic domain, which is rich in valines (V), leucines (L) and isoleucines (I). Asterisks indicate charged amino acids.

(Haas-Stapleton *et al.*, 2004). Complementation assays revealed that the defect in oral infectivity of P74-null AcMNPV could be rescued by mixed infection of *p74*-null virus with wt AcMNPV (Zhou *et al.*, 2005) or purified P74 protein (Yao *et al.*, 2004). PIF-1 was first identified in *Spodoptera littoralis nucleopolyhedrovirus* (Kikhno *et al.*, 2002). The virus with deletion of *pif-1* (Spli7) was shown to be unable to infect *S. littoralis* larvae *per os* and the product of *pif-1* is an ODV-specific structural protein (Kikhno *et al.*, 2002). PIF-2 was first identified in SeMNPV, where deletion of *pif-2* (*Se35*) resulted in the complete loss of *per os* infectivity to the host (Pijlman *et al.*, 2003). It was speculated that *pif-2* from SeMNPV would encode a structural protein of ODV (Pijlman *et al.*, 2003). Recent research by Ohkawa *et al.* (2005) revealed that PIF-1 (Ac119), PIF-2 (Ac022) and PIF-3 (Ac115) are essential for oral infection of AcMNPV. The competing assay implicated that PIF-1 and PIF-2 might function as attachment proteins for ODV binding to primary target cells in the midgut, while PIF-3 mediates another unidentified, but critical, early event during primary infection (Ohkawa *et al.*, 2005). In this study, we have ascertained that the homologue of *pif-2* in HearNPV, *Ha132*, encodes a structural component associated with ODVs and that the deletion of *Ha132* resulted in the complete elimination of *per os* infectivity of OBs. Therefore, like MNPVs, PIF-2 is also an essential factor for oral infection in SNPVs. It was speculated that P74, PIF-1 and PIF-2 might interact with each other (Kikhno *et al.*, 2002; Pijlman *et al.*, 2003) and with microvillar binding partners (Ohkawa *et al.*, 2005). Further experiments would allow the determination of possible interactions of these ODV-specific structural proteins and how these proteins enable ODV to set up a successful infection *in vivo*.

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