

Helicoverpa armigera Nucleopolyhedrovirus ORF80 Encodes a Late, Nonstructural Protein

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The *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) ORF80 (*ha80*) has 765 bp encoding a protein with approximately 254 amino acids and a predicted molecular weight of 30.8 kDa. Homologues of *ha80* are found in most baculovirus sequences, including those from lepidopteran NPVs, lepidopteran granuloviruses (GVs), hymenopteran baculoviruses, and one dipteran baculovirus, yet their functions remain unclear. In this study we characterized *ha80*, and showed that it was transcribed late in infected host cells (HzAM1). The product of *ha80* was a 31 kDa protein that was not a structural protein of budded virus (BV) or occlusion-derived virus (ODV) particles. Ha80 was first detected in the cytoplasm of infected HzAM1 cells at 12 h p.i., and was observed in the nucleus at later stages of infection, suggesting that it may be involved in transporting viral proteins into the host cell nucleus or play its roles in the nucleus.

Keywords: Cellular localization, Expression, HearNPV *orf80*, *Helicoverpa armigera*, Nucleopolyhedrovirus, Transcription

Introduction

Baculoviridae is a large family of viruses that infects invertebrates, particularly insects from the order Lepidoptera. Baculoviruses are natural regulators of insect populations in agriculture and forestry, and have been used as bioinsecticides to control insect pests world-wide. The baculoviruses are divided into two genera based on their occlusion body morphology, the Nucleopolyhedroviruses (NPVs), which have large occlusion bodies containing numerous virions, and the Granuloviruses (GVs), which harbor a single virion inside small granular

occlusion bodies (Rohmann, 1999; Winstanley and O'Reilly, 1999). *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearNPV) belongs to the NPV genus and infects only two hosts, *Helicoverpa armigera* and *Helicoverpa zea*. These two insects are economically important polyphagous pests in both Asia and America, responsible for considerable damage to many vegetables and field crops. HearNPV is successfully used as a large scale commercial biological insecticide against cotton worm in China (Zhang, 1981).

The sequences of two HearNPV isolates, G4 and C1, have been determined (Chen *et al.*, 2001; Zhang *et al.*, 2005), and a number of HearNPV genes are characterized, including *lef2* (Chen *et al.*, 1999), *Ha29* (Guo *et al.*, 2005), *p10* (Dong *et al.*, 2005), *FP25K* (Wu *et al.*, 2005), *Bv-e31* (Wang *et al.*, 2005), *Ha101* (An *et al.*, 2005), *Ha135* (An *et al.*, 2005), *Ha2* (Nie *et al.*, 2006), *Ha133* (Long *et al.*, 2006), *Ha128* (An *et al.*, 2006), *Ha39* (Xu *et al.*, 2006) and *Ha83* (Wang and Zhang, 2006). However, the functions of many ORFs remain unknown.

HearNPV ORF80 (*ha80*) is located between 72,915 and 73,679 bp in the HearNPV-C1 genome, encodes a putative 254 aa protein with a predicted molecular weight of 30.8 kDa, and is transcribed in the opposite orientation to the polyhedrin gene. *Ha80* is highly conserved, sharing homology with all completely sequenced baculovirus genomes, including lepidopteran NPVs, lepidopteran GV, hymenopteran baculoviruses, and one dipteran baculovirus. This gene is one of the thirty core baculovirus genes, yet its function remains unknown.

In this study, we analyzed the putative ORF80 of HearNPV and examined expression and transcription of *ha80* in HearNPV infected HzAM1 cells. We found that the Ha80 protein was synthesized late in infection showed by confocal microscopy that it is present in the cytoplasm at 12 h p.i., and observed in the nucleus at later stages of infection.

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Materials and Methods

Viruses, insects, and cells. HearNPV C1 was used for infections and propagated in the *Helicoverpa zea* cell line, HzAM1 (McIntosh, 1983). HzAM1 cells were grown in TNM-FH insect medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco). A culture of *H. armigera* larvae was maintained for HearNPV polyhedra production as described by Sun *et al.* (1998). Routine methods for baculovirus manipulations were described by O'Reilly *et al.* (1992).

Computational analyses. The gene *ha80* and deduced amino acid sequence were analyzed using the Gentyx-Win (Ver. 5) (Software Development Co.) and DNASTAR programs (Ver. 5.02) (DNASTAR, Inc.). Predictions of the domains, motifs, signal sequence, and post-translational modifications were made using 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 BLAST and PSI-BLAST programs (Altschul *et al.*, 1997), and sequence alignment was performed using Clustal-W (<http://www.ebi.ac.uk/clustalw/>) and edited by GeneDoc (Ver. 2.04) (Free Software Foundation, Inc.).

Transcriptional analysis. Total RNA was isolated from HzAM1 cells infected with HearNPV strain C1 (M.O.I. of 5 TCID₅₀ units per cell) at 0, 3, 6, 12, 24, 36 and 48 h p.i. using Trizol (Life Tech.) according to the manufacturer's protocol, and RNA concentration was determined at an absorbance of 260 nm. Radiolabeled probes were prepared from the Prime-a-Gene Labeling System Kit (Promega), according to the manufacturer's protocol. RNA was denatured by heating at 65.8°C for 15 min. For Northern blot analysis, 10 mg of total RNA (each lane) was separated by denaturing gel electrophoresis using 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). The membranes were prehybridized in ULTRAhyb hybridization Buffer for 3 h at 42°C. The (³²P)-dCTP labeled probe was added to the hybridization solution and incubated at 42°C for 18 h. The membranes were washed twice in 2 × SSC, 0.1% SDS at 42°C, and once in 0.1 × SSC, 0.1% SDS at 42°C, before exposure to X-ray film.

Generation of anti- Ha80 antiserum. The complete HearNPV ORF80 (765nt) was amplified by PCR using an upstream primer (5'-AGGATCCATGATTCCGTTGACACCT-3') incorporating a *Bam*HI site (underlined) and a downstream primer (5'-CAAGCTTTTATTTTAATTGTAATAG-3') with a *Hind*III site (underlined). The amplified fragment was inserted into pGEM-T easy vector (Promega). The insertion was retrieved by digestion with *Bam*HI and *Hind*III, and subcloned into the expression vector, pET-28a (Novagen), in-frame with the C-terminus of the 6 × His tag in this plasmid. The recombinant plasmid, P28a-*ha80*, was transformed into *Escherichia coli* BL21 (DE3) cells and fusion protein expression was induced by incubation with 1 μM IPTG after the optical density at 600 nm had reached ~0.7. The His-tagged Ha80 fusion protein was confirmed by staining with goat monoclonal anti-His antibody (Amersham Pharmacia). The recombinant Ha80

protein was purified according to the Protein Purification System protocol (Invitrogen), using a Ni²⁺ nitrilotriacetic acid-agarose column, and used as an immunogen to raise Ha80-specific antiserum in male rabbits. Five injections with 200 μg of purified 6 × His-Ha80 fusion protein were administered to each rabbit. For the first and second injections, complete Freund's adjuvant (Sigma) and incomplete adjuvant (Sigma) were used with the fusion protein, respectively. The rabbits were boosted on day 35, 49, and 63. Ten days after the last injection, the rabbits were bled by cardiac puncture.

Western blot analysis. A monolayer of HzAM1 cells was cultured and infected with HearNPV at an M.O.I. of 5 TCID₅₀ units per cell. Cells were harvested at 0, 3, 6, 12, 24, 36 and 48 h p.i., pelleted at 5,000 g, resuspended in 1 × PBS, and lysed in SDS-PAGE loading buffer by boiling for 10 min. Protein samples were separated by SDS-PAGE, transferred onto an Immobilon-P nitrocellulose membrane (Millipore) by semi-dry transfer cell (Trans-Blot SD) according to the manufacturer's suggested procedure, and incubated overnight in 2% skimmed milk powder in TBS at 4°C. The membrane was allowed to react with 1 : 5,000 Ha80 antiserum for 1 h at room temperature and treated as previously described by IJkel *et al.* (2001). Immunoreactive proteins were visualized using goat anti-rabbit IgG-HRP (Southern Biotech) according to the manufacturer's protocol. Antisera against the two structural proteins, BV-e31 (Wang *et al.*, 2005) and ODV-EC43 (Fang *et al.*, 2003), were used as positive controls for immunodetection of the Ha80 protein in BV and ODV.

Purification of the HearNPV ODV and BV fractions. BV was purified from harvested supernatants of infected cells and ODV was purified from polyhedra as described by O'Reilly *et al.* (1992). For analysis of structural proteins, BV and ODV were isolated from their stocks by pelleting through a 20% sucrose cushion and centrifuging on a 25 to 60% sucrose gradient. After purification, BV was incubated with 1% Nonidet-P40 and fractions containing the envelope or capsid were separated by centrifugation (Guarino *et al.*, 1995).

Confocal laser scanning microscopy. HzAM1 cells were grown in plastic petri dishes and infected with wild-type HearNPV. At different time points, the cells were washed three times in 1 × PBS, fixed with 4% paraformaldehyde in 1 × PBS, permeabilized for 10 min with 0.2% Triton X-100, and allowed to react with 1 : 2000 polyclonal antiserum and protein G fused to enhanced green fluorescent protein (EGFP), using a method previously described by Spector *et al.* (1998). The cells were examined using a Zeiss LSM510 confocal laser-scanning microscope for fluorescence detection.

Results

Sequence analysis. *Ha80* is 765 nucleotides (nt) long and encodes a protein with approximately 254 aa and a predicted size of 30.8 kDa (Fig. 1). The baculovirus consensus late promoter motif, ATAAG, was found at 189 nt, and a TATA box was found at 208 nt upstream of the start codon, ATG, indicating that *ha80* may be expressed at a later stage of the infection cycle. One polyadenylation signal sequence, AATAAA,

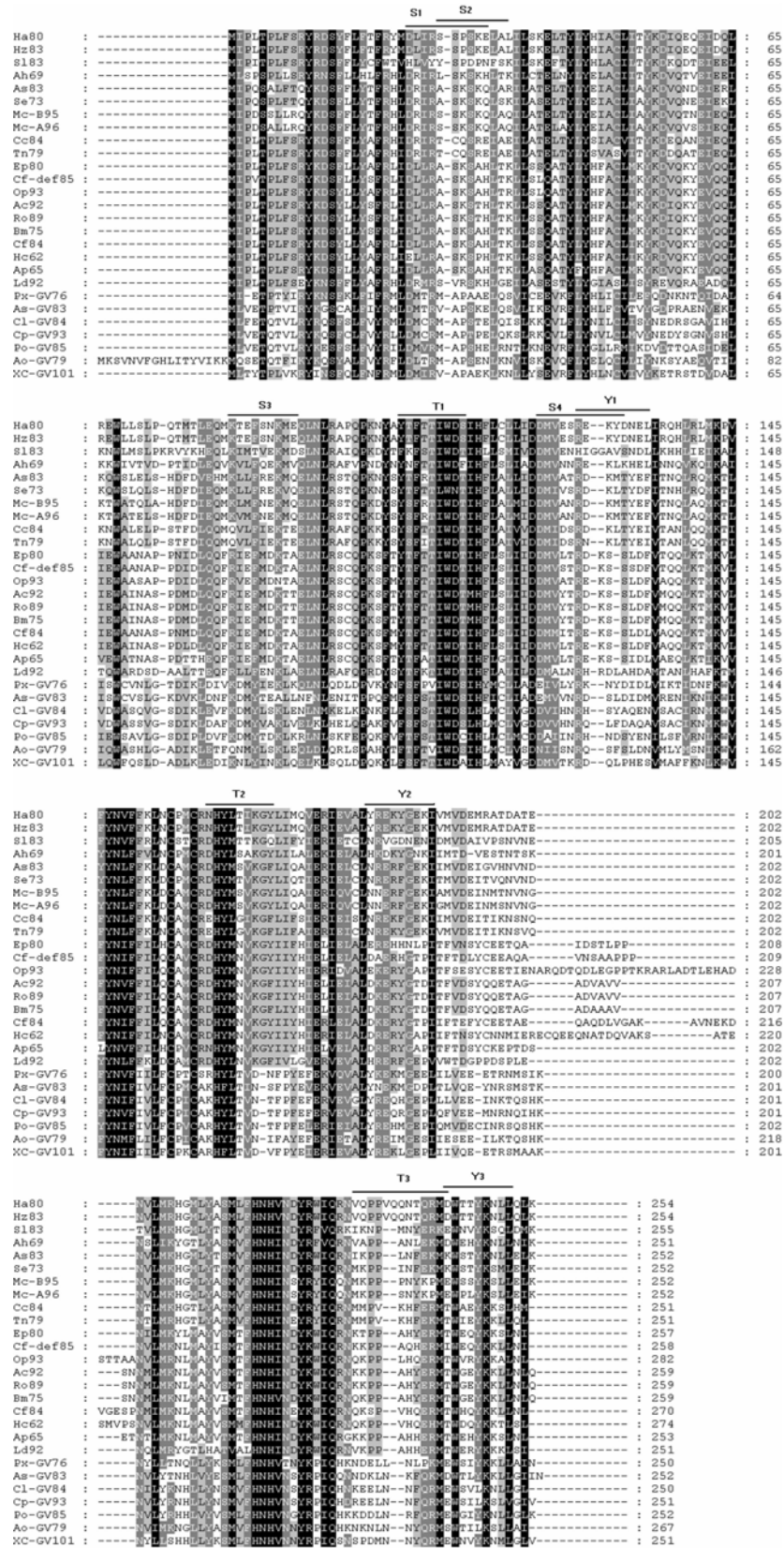


Fig. 1. Sequence alignments of Ha80 and its homologs that share the identities more than 30%. Black shade: 100% identity. Grey shade: 80% identity. The putative serine phosphorylation sites, threonine phosphorylation sites and tyrosine phosphorylation sites are denoted with S(1-4), T(1-3) and Y(1-3), respectively.

Table 1. Homologous ORFs of HearNPV ORF80 among other baculovirus genomes

NPV/GV	Homologues	aa identities (%)	GenBank accession
Lepidopteran NPV	<i>Helicoverpa zea</i> NPV <i>orf83</i>	100	AAL56089
	<i>Agrotis segetum</i> NPV <i>orf83</i>	60	YP_529753
	<i>Spodoptera exigua</i> NPV <i>orf73</i>	59	AAF33602
	<i>Chrysodeixis chalcites</i> NPV <i>orf84</i>	58	YP_249688
	<i>Trichoplusia ni</i> NPV <i>orf79</i>	58	AAZ67449
	<i>Mamestra configurata</i> NPV <i>B orf95</i>	57	NP_689270
	<i>Mamestra configurata</i> NPV <i>A orf96</i>	56	AAQ11115
	<i>Adoxophyes honmai</i> NPV <i>orf69</i>	54	NP_818716
	<i>Bombyx mori</i> NPV <i>orf75</i>	52	AAC63761
	<i>Spodoptera litura</i> NPV <i>orf83</i>	52	AAL01765
	<i>Autographa californica</i> NPV <i>orf92</i>	51	NP_047492
	<i>Rachiplusia ou</i> NPV <i>orf89</i>	51	AAN28076
	<i>Antheraea pernyi</i> NPV <i>orf65</i>	49	YP_611033
	<i>Choristoneura f. defective</i> NPV <i>orf85</i>	49	NP_932694
	<i>Epiphyas postvittana</i> NPV <i>orf80</i>	49	AAK85644
	<i>Lymantria dispar</i> NPV <i>orf92</i>	49	AAC70280
	<i>Choristoneura fumiferana</i> NPV <i>orf84</i>	48	NP_848395
	<i>Hyphantria cunea</i> NPV <i>orf62</i>	47	YP_473250
<i>Orgyia pseudotsugata</i> NPV <i>orf93</i>	47	AAC59092	
<i>Leucania separata</i> NPV <i>orf92</i>	28	BAA24234	
Lepidopteran GV	<i>Xestia c-nigrum</i> GV <i>orf101</i>	43	AAF05215
	<i>Phthorimaea operculella</i> GV <i>orf85</i>	42	AAM70283
	<i>Agrotis segetum</i> GV <i>orf83</i>	40	YP_006261
	<i>Cryptophlebia leucotreta</i> GV <i>orf84</i>	40	NP_891931
	<i>Plutella xylostella</i> GV <i>orf76</i>	39	AAG27374
	<i>Cydia pomonella</i> GV <i>orf93</i>	38	AAK70753
	<i>Adoxophyes orana</i> GV <i>orf79</i>	37	AAP85716
Hymenopteran baculovirus	<i>Neodiprion sertifer</i> NPV <i>orf24</i>	26	YP_025131
	<i>Neodiprion lecontei</i> NPV <i>orf16</i>	23	YP_025213
Dipteran baculovirus	<i>Culex nigripalpus</i> baculovirus <i>orf14</i>	15	NP_203317

overlapped with the stop codon, TAA. Two consensus eukaryotic transcription factor-binding site motifs were found 72 and 97 nt upstream of the ATG.

Ten phosphorylation sites were predicted in the putative Ha80 protein using the NetPhos 2.0 Server, including four serine phosphorylation sites (aa 24-34, 27-36, 82-87 and 122-131), three threonine phosphorylation sites (aa 104-113, 160-169 and 234-244), and three tyrosine phosphorylation sites (aa 126-135, 181-190 and 243-252) (Fig. 1). No other domain, signal peptide, transmembrane region, mitochondrial targeting sequence, nuclear localization signal, or membrane retention signal were found by other motif search engines.

The protein databases, GenBank and SWISS-PROT, showed that the putative Ha80 protein was homologous to the products of 23 NPV ORFs and seven GV ORFs. Alignment of the Ha80 sequence with its NPV and GV homologues was done using Clustal W (Table 1). A homologue from a possible variant of HearNPV, *H. zea* NPV (Hz83), shared 100% sequence identity with Ha80. Homologues from the 22 other NPVs

shared 28-60% identity with Ha80, while the homologues from seven lepidopteran GVs shared 37-43% identity with Ha80. Unexpectedly, an Ha80 homologue from a lepidopteran NPV, *L. separata* NPV, shared less identity with Ha80 than its homologues from GV. Homologues from two hymenopteran baculoviruses, *N. sertifer* NPV (ORF24) and *N. lecontei* NPV (ORF16), shared only 26% and 23% homology with Ha80, respectively, while the homologue from the dipteran baculovirus, *Culex nigripalpus* baculovirus (ORF14), shared 15% with Ha80, the lowest sequence identity.

Transcriptional analysis. The temporal regulation of *ha80* transcription was analyzed by Northern blot. A single transcript of ~0.8 kb was detected at 12 h p.i., and transcription increased until 36 h p.i. (Fig. 2), suggesting that Ha80 is produced late in infection.

Time course of *Ha80* expression in HearNPV-infected cells. To study *ha80* expression and function, we produced a

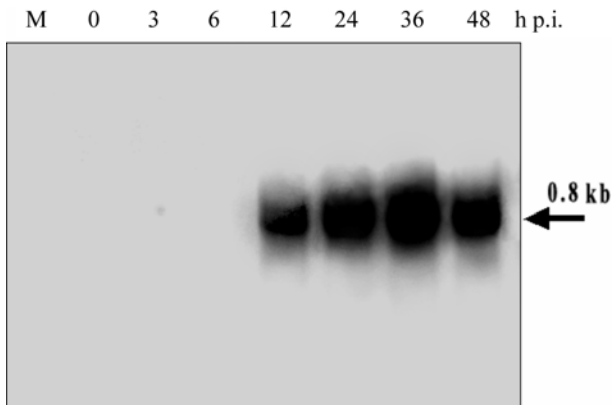


Fig. 2. Northern blot analysis of temporal regulation of transcription for *ha80* gene performed on total RNA extracted from HearNPV-infected HzAM1 cells. The size of specific hybridization bands is indicated on the right with an arrow. Times p.i. are indicated above the lanes (M, mock infected).

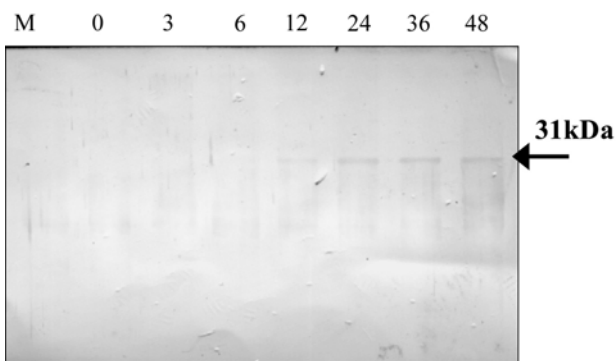


Fig. 3. Time course of *ha80* product by western blot analyses. HearNPV-infected HzAM1 cells were harvested at the indicated times post infection. M indicates mock infection. The estimated size of detected bands is indicated on the right with an arrow.

polyclonal antibody against the Ha80 protein by immunizing rabbits with a purified His-tagged Ha80 fusion protein. HearNPV-infected HzAM1 cells were harvested at different time points and probed with the Ha80 polyclonal antibody by

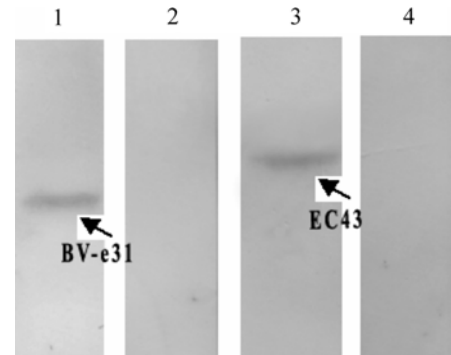


Fig. 4. Immunodetection of BV and ODV proteins. Lane 1 and 2 are western blot analyses for BV-e31 with BV-e31 antiserum and Ha80 with Ha80 antiserum on BV, respectively. Lane 3 and 4 are western blot analyses for ODV-EC43 with ODV-EC43 antiserum and Ha80 with Ha80 antiserum in ODV, respectively.

western blot. An immunoreactive band of approximately 31 kDa was first observed at 12 h p.i. and remained detectable until 48 h p.i. (Fig. 3). This band was consistent with the predicted molecular weight of Ha80 (30.8 kDa). The time course of Ha80 protein expression correlated with *ha80* gene transcription, confirming that Ha80 is synthesized in the late stage of the infection cycle.

Immunodetection of the Ha80 protein in BV and ODV. To investigate whether Ha80 is a structural protein, western blot analysis was carried out on HearNPV BVs and ODVs. While specific bands were detected in BVs and ODVs using Bv-e31 antiserum and ODV-EC43 antiserum, respectively, no positive bands could be detected with Ha80 antiserum (Fig. 4). Thus, Ha80 is a non-structural protein.

Localization of the Ha80 protein in infected host cells. Localization of the Ha80 protein in host cells was analyzed by fluorescence detection using confocal laser-scanning microscopy (Fig. 5). The results showed that Ha80 protein was localized in the cytoplasm of infected HzAM1 cells at the early stage of translation (from 12 h p.i.), and present in the nucleus at later stages of infection (48 h p.i.).

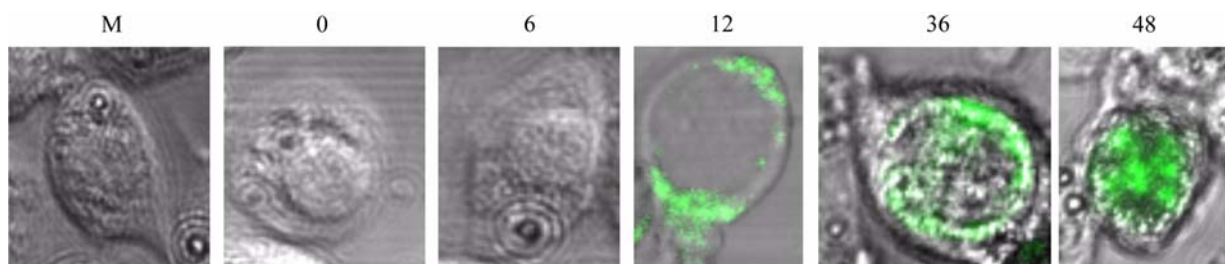


Fig. 5. Localization of Ha80 protein in infected host cells. The fluorescence detection using confocal laser-scanning microscopy for cells harvested at mock infected, 0, 6, 12, 36 and 48 h p.i., respectively.

Discussion

In this report, we analyzed the transcriptional expression patterns of *ha80*. *Ha80* is expressed as a late gene, and encodes a non-structural protein that has not been previously characterized in any other baculoviruses.

Homologues of *ha80* are present in all 30 completely sequenced baculoviruses, including the lepidopteran NPV, lepidopteran GV, hymenopteran baculoviruses, and one dipteran baculovirus, suggesting that they were acquired prior to the divergence of these genera. The homologue with the highest identity was *hz83* from HzSNPV, and the homologous with the lowest identities were found in the dipteran baculovirus, and two hymenopteran baculoviruses. These findings suggest that *ha80*-like genes are important for the infection cycle of all baculoviruses.

Ha80 localization experiments indicated that Ha80 protein is produced in the cytoplasm at 12 h p.i. and transported into the nucleus by 48 h p.i.. Similar protein transportation patterns were also found in baculovirus genes that are involved in viral infection of host insect larvae, such as BV/ODV-E26, BV/ODV-C42 and ODV-E66 (Beniya *et al.*, 1998; Hong *et al.*, 1997). Nuclear transportation of these proteins is mediated by FP25K (Braunagel *et al.*, 1999). Like Ha83 (Wang *et al.*, 2006), Ha80 may be involved in the transportation process by interacting with one of the proteins that undergoes nuclear translocation.

In conclusion, sequencing, northern blot, western blot, and cellular localization analyses indicated that *ha80* is a functional gene in HearNPV that may play its roles in the nucleus or associate with proteins required for nuclear transport in host cells. Further studies are necessary to determine the specific mechanism of *ha80* function.

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