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# Characterization of *pif*, a gene required for the *per os* infectivity of *Spodoptera littoralis* nucleopolyhedrovirus

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During plaque purification of *Spodoptera littoralis* nucleopolyhedrovirus in *S. littoralis* SI52 cell culture, a deletion mutant virus was isolated. Analysis of the biological properties of this mutant virus revealed an absence of *per os* infectivity of the occluded virus. Infectivity by injection of the non-occluded (budded) virus is not different between the wild-type and the deleted virus. Restriction analysis of the mutant virus genome revealed a 4.5 kb deletion within the *NotI* D fragment. The observed phenotype was mapped to the deleted region by rescue experiments. The deletion was characterized and the equivalent DNA fragment on the wild-type virus was sequenced. By co-transfecting the DNA of the deleted virus with plasmids derived from the wild-type virus, it was possible to determine that ORF 7 in this fragment is responsible for the observed phenotype. ORF 7, called *pif* (*per os* infectivity factor), is homologous to ORF 119 of *Autographa californica* nucleopolyhedrovirus. Similar ORFs are present in all sequenced baculoviruses. The product of this gene is an occlusion body-derived virion structural protein required only for the first steps of larva infection, as viruses being produced in cells expressing the gene but not containing it in their genomes are able to produce successful infections.

## Introduction

Baculoviruses are large DNA viruses that infect arthropods, mainly members of the order Lepidoptera. In their life cycle, they produce two kinds of particles, a budded, non-occluded virus (BV), which buds out of the infected cell and is responsible for the cell-to-cell transmission of the virus, and an occluded form, the occlusion body (OB), which is responsible for protecting the virus between encounters with larvae (Funk *et al.*, 1997). A variable number of virions are included in the paracrystalline structure of the OB, mainly constituted by the virus-encoded polyhedrin protein; these virions are called occlusion body-derived virions or ODVs. In some baculoviruses, known previously as multiple nucleopolyhedroviruses (MNPVs), ODVs can be formed by more than one nucleocapsid (that is, a single ODV includes several complete genomes).

Both forms of virus are usually produced in a sequential manner in the same cells: first BVs are produced, allowing the virus to spread to all susceptible tissues in the larvae; later, the virions are retained within the nuclei where they acquire

specific proteins and are eventually included into the OB. OBs will only be released after the death of the larva.

Although the virions present in both forms differ in some of their virus-derived proteins and in the composition of their viral membranes (Braunagel & Summers, 1994), they contain the same genome. The two phenotypes play different roles in the virus cycle. BVs are responsible for cell-to-cell infection within the host, while OBs ensure the propagation of the virus between hosts.

The differences in the tissue tropism and the ability of the two virus phenotypes to survive in the natural environment can be related to the specific proteins differentially associated with each type of virion. For instance, the BV of *Autographa californica* nucleopolyhedrovirus (AcMNPV) contains the GP64 protein, which is required for cell-to-cell transmission and which is not present in ODVs (Monsma *et al.*, 1996). The absence of the polyhedrin, the major OB protein in the NPV, severely impairs virus survival in the environment but does not affect their *in vitro* maintenance (Smith *et al.*, 1983).

The life cycle of baculoviruses in nature starts with the ingestion of OBs present on contaminated diet by a larva. The OBs are dissolved in the midgut and the virions they contain are released. The ODVs pass through the peritrophic membrane and infect the midgut cells. In some baculoviruses, virus

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enhancing factors (VEFs) present in the OBs increase infectivity (Bischoff & Slavicek, 1997; Tanada, 1985; Wang *et al.*, 1994).

Infection of the larval midgut cells occurs by direct fusion of the ODV envelope to the plasma membrane (Granados & Lawler, 1981). Horton & Burand (1993) analysed the entry of *Lymantria dispar* nucleopolyhedrovirus (LdMNPV) ODVs into *L. dispar* cells in culture (IPBL-LdEIta) and in brush-border membrane vesicles. They deduced that the entry of the virus could occur in two stages: binding of ODVs to the cell membrane followed by fusion, mediated by ODV attachment and fusion factors, respectively.

Knock-out experiments demonstrate that the P74 protein of AcMNPV, the expression product of AcMNPV ORF 138 (Ayres *et al.*, 1994), is required for the infectivity of ODVs (Kuzio *et al.*, 1989; Faulkner *et al.*, 1997), suggesting its involvement in the attachment to a specific receptor. This protein appears to be conserved among all sequenced baculoviruses.

The analysis of viral genes involved in the initial stages of virus entry into the host would greatly increase our understanding of this process. It would also enable the determination of what host defence mechanisms, if any, operate in the insect midgut.

In this report we show that a second protein, encoded by ORF 7 of the *NotI* D fragment of *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV), is required for the infectivity of the OBs. ORF 7 is homologous to ORF 119 of AcMNPV (Ayres *et al.*, 1994). This ORF is also conserved in all sequenced baculoviruses. We demonstrate that it is a structural protein of the ODV envelope required only in the first steps of *per os* infection.

## Methods

■ **Viruses, cells and larvae.** The wild-type SpliNPV strain M2 (Croizier *et al.*, 1989) was used as a reference virus. A deletion mutant, SpliNPV  $\Delta 4$ , appeared in our stocks during a cloning procedure while studying the *egt* region (G. Croizier, L. Croizier and M. López Ferber, unpublished results). This mutant was cloned by plaque assay.

SpliNPV cell culture infections, co-transfections and titrations were done in *S. littoralis* S152 cells cultured in a modified TC100 medium supplemented with 10% FCS (Croizier *et al.*, 2000). AcMNPV recombinants were obtained and amplified on *S. frugiperda* Sf9 cells. BV titres in cell culture supernatants were estimated by the end-point dilution method according to Summers & Smith (1987).

Biological tests were carried out using *S. littoralis* larvae obtained from our laboratory colony. All larvae, healthy or infected, were reared on semi-synthetic medium (Poitout & Bues, 1974) at 23 °C, 70% relative humidity and with a photoperiod of 16 h.

■ **Purification and isolation of OBs and ODVs.** Healthy, third instar (L3) *S. littoralis* larvae were injected through a proleg with a  $0.45 \pm 12$  mm needle. Each larvae received 8  $\mu$ l of ODV solution or infectious S152 cell culture supernatant diluted in TG3 medium. After injection, larvae were reared individually under the conditions indicated above. Dead larvae were processed as described previously (Croizier & Ribeiro, 1992). After brief sonication to disrupt aggregates, the

concentration of OBs was calculated using an improved Neubauer haemocytometer as outlined by Hunter-Fujita *et al.* (1998). To liberate ODVs, aliquots of  $5 \times 10^9$  OBs were incubated in 1 ml of dissociation buffer (50 mM Na<sub>2</sub>CO<sub>3</sub>) at room temperature for 40 min, followed by 2 min of centrifuging at 13 000 r.p.m. in a microfuge. The pellet was re-suspended in 0.5 ml of dissociation buffer and centrifuged again. The combined supernatants were layered onto a 14 ml, 20–60% (w/w) sucrose–TE gradient and centrifuged at 18 000 r.p.m. for 1 h at 4 °C. The multiple ODV bands were removed, diluted in TE, pelleted and finally re-suspended in  $0.1 \times$  TE (Croizier *et al.*, 1980).

■ **Bioassays.** The droplet feeding technique was used for all *per os* tests on neonates and L3 larvae (Hughes & Wood, 1981). Lots of 48 larvae were either fed with five logarithmic suspensions of OBs to estimate the LD<sub>50</sub> or injected as indicated above.

After *per os* infection or injection, larvae were reared individually in conditions similar to the non-infected larvae. Dead larvae were examined to determine the presence of OBs. OBs were purified from each group of dead larvae. Viral DNA was extracted from the larvae and submitted to restriction endonuclease analysis to assess its identity.

The infectivity of ODVs by injection was tested on groups of 20 L3 larvae. The larvae were reared as stated previously.

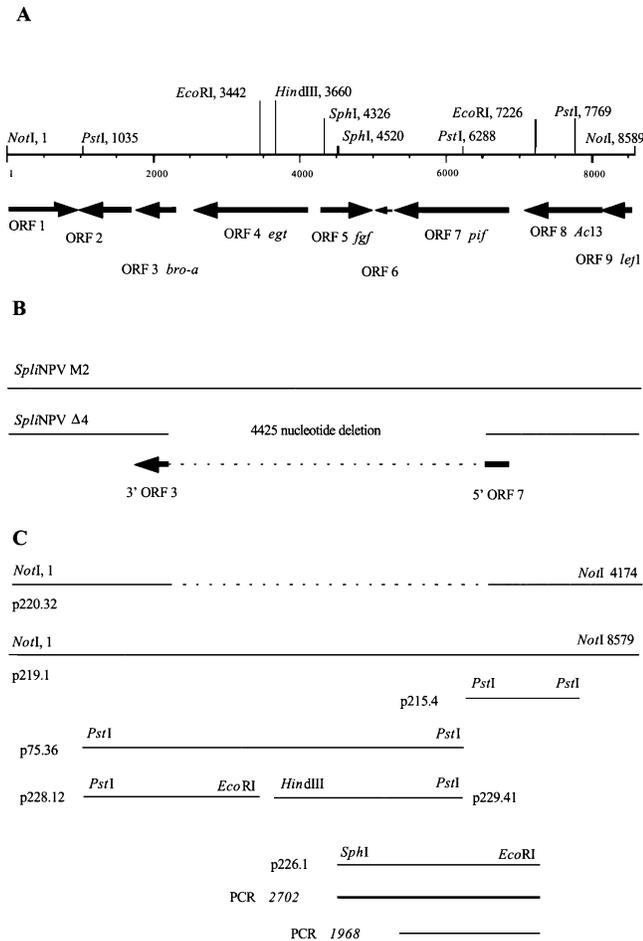
■ **Plasmids and PCR fragments.** The wild-type *NotI* D restriction fragment was cloned into a pBluescript plasmid (Stratagene) to give p219.1 (Fig. 1). Similarly, the *PstI* H and K restriction fragments were cloned into pUC19 to give p75.36 and p215.4, respectively. Plasmids p226.1, p228.12 and p229.41 were constructed by inserting into pUC19 the appropriate restriction fragments from p219.1 or p75.36 (Fig. 1). The *NotI* restriction fragment encompassing the deletion in the SpliNPV  $\Delta 4$  virus was similarly cloned to give p220.32. Both *NotI* D fragments from the wild-type virus (insert of p219.1, 8.6 kb) and the SpliNPV  $\Delta 4$  deletion mutant (insert of p220.32, 4.2 kb) were sequenced by the dye terminator method on an ABI 373 automatic sequencer.

■ **Complementation analysis.** To test for the rescue of infectivity,  $1 \times 10^5$  S152 cells were transfected with 800 ng of SpliNPV  $\Delta 4$  DNA alone or mixed with different plasmids (50  $\times$  molar ratio to the viral DNA) covering the deleted region (Fig. 1) using DOTAP (Roche). Co-transfected cells were harvested once the OBs were produced (10 days after infection). Cells were then rinsed with PBS and re-suspended in 10–20  $\mu$ l PBS. These samples were then frozen, both to inactivate any BV that could be present and to disrupt the cells to liberate the OBs. The OB solution was given to neonate larvae using the droplet feeding method.

Larva that had fed on the solution over a 5 min period were isolated in rearing boxes with feeding medium. Mortality was recorded daily. Dead larvae were checked for OBs and the viral DNA was extracted, digested with endonucleases and analysed by electrophoresis. OBs were further passaged to test for *per os* infectivity.

PCR amplifications were performed on p226.1 DNA using the Expand High Fidelity Polymerase reagent (Roche). For PCR 2702, the universal forward and reverse primers were used to amplify the complete p226.1 insert. For PCR 1968, the primers used were the universal forward primer and oligonucleotide SpliNPVORF7/1 (5' cgtacgctcaggcagctgctg-caggctaacgaacgctatagttgggtacgtg 3'), which hybridizes to the last codons of ORF 7. As a negative control, a tube containing similar amounts of template, but which was not subjected to PCR, was used in each co-transfection. A 1:100 of the PCR product was used for each co-transfection, after digestion with *SphI*/*EcoRI*.

■ **Construction of recombinant viruses.** The complete SpliNPV *NotI* D ORF 7 was amplified by PCR from p219.1 DNA. Two different PCR products were obtained encoding the complete ORF either with or without a 6  $\times$  (His)-tag coding sequence at their 3' end, flanked by *BgIII*



**Fig. 1.** Arrangement of ORFs within restriction fragment *NotI* D of *SpliNPV* and complementation analysis strategies. (A) Organization of *SpliNPV* M2 in the *NotI* D restriction fragment. ORFs are numbered from left to right and their names are indicated. (B) Comparison between the *NotI* D restriction fragments of *SpliNPV* M2 and *SpliNPV* Δ4. The composite ORF arising from the fusion of *pif* and *bro-a* is marked. (C) *SpliNPV* Δ4 M2 DNA fragments used for sequencing (p219.1 and p220.32) and in the complementation strategies are shown. The letter 'p' followed by a number indicates a plasmid. The restriction sites at the extremities of the viral DNA sequences are noted for each plasmid. PCR fragments containing the complete *pif* coding sequence were obtained using p226.1 as template and either the universal and reverse primers (PCR 2702) or the universal primer and primer *SpliNPVORF7/1* (PCR 1968).

sites. A single primer was used for the 5' ends (ORF7-*BgIII*, 5' gctgaagatctatgtacaagatactattgatag 3') and two specific primers for the 3' ends (ORF7-*His-BgIII*, 5' agctagatctctagtggtggtggtggtgacgaacgctatagtttgggtacgtg 3', and ORF7-*BgIII*, 5' agctagatctctaaacgaacgctatagtttgggtacgtg 3'). PCR products were cloned into the pAcUW21 vector (López Ferber *et al.*, 1995) under the AcMNPV p10 promoter to give p252 (ORF 7 with a histidine tag) and p253 (native ORF 7). Recombinant viruses BacPAKPIFHIS and BacPAKPIF were obtained by co-transfection on Sf9 cells of BacPAK6 DNA (Kitts & Possee, 1993) and plasmids p252 and p253, respectively. Recombinant viruses were selected for their occlusion-positive phenotype by three rounds of plaque purification.

**Antibodies, SDS-PAGE and Western blot analysis.** Sequence analysis predicted that the regions between aa 163 and 179 and 429 and 442 of the deduced protein sequence of the *SpliNPV* *NotI* D fragment

ORF 7 would be the most antigenic regions. Synthetic peptides were made against these two regions (*Spli7-1* and *Spli7-2*, respectively), coupled to KLH and used to raise antibodies in rabbits (Ab262 and Ab263).

To detect the histidine tag, a monoclonal mouse anti-His antibody (Ab His) was used (Eurogentec). AcMNPV GP67 was detected using AcV<sub>5</sub> monoclonal antibody (Hohmann & Faulkner, 1983).

Vertical slab SDS-PAGE was performed using a 3.5% stacking gel and a 10% separating gel. Before loading, samples were boiled for 10 min in 50 mM Tris-HCl, pH 6.8, 2% SDS, 1% β-mercaptoethanol and 10% glycerol. Proteins were transferred onto PVDF membranes (Roche). The membranes were blocked with TBST [0.9% NaCl (w/v), 10 mM Tris-HCl, pH 8.0, and 0.1% Tween 20] and 5% dry skimmed milk. Antibodies were allowed to bind overnight at 4 °C (Ab262, 1:3000 dilution; AbHis, 1:1500 dilution). Blots were washed four times (15 min) with TBST and incubated for 1 h with either horseradish peroxidase-linked anti-rabbit antibodies, 1:500 dilution (Sanofi Pasteur Diagnostics), or alkaline phosphatase-linked anti-mouse antibodies, 1:2000 dilution (Santa Cruz Biotechnology). Blots were washed four times with TBST and developed using chemiluminescence (Roche).

**Glycosylation analysis.** Sf9 cells ( $5 \times 10^6$ ) were infected with BacPAKPIF. At 120 h post-infection, cells were collected, pelleted, washed with PBS and re-suspended in 300 μl PBS. Cells were disrupted by three freeze-thaw cycles followed by passage through a 26 gauge needle. The particulate material, containing the ORF 7 gene product was pelleted for 5 min at 13000 r.p.m. A sample of 3 μl of concentrated BacPAKPIF BV suspension (10 μg of protein) was added to the pellet to provide an internal AcMNPV GP67-based positive control. After adding 50 μl of 5% SDS, the pellet was boiled for 10 min and the insoluble fraction was removed by centrifuging for 5 min at 13000 r.p.m. The supernatant was then diluted 2.5 times with water and distributed in 10 μl aliquots. Each 10 μl aliquot was mixed with an equal volume of 2 × treatment buffer (40 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM EDTA, 2% NP-40 and 2% β-mercaptoethanol, pH 7.2) and either 2 units of F-endoglycosidase (Roche) or 2 μl of water as negative control, and incubated for 16 h at 37 °C. The proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with Ab262 (1:2000 dilution) or AcV<sub>5</sub> (1:2000 dilution) (Hohmann & Faulkner, 1983).

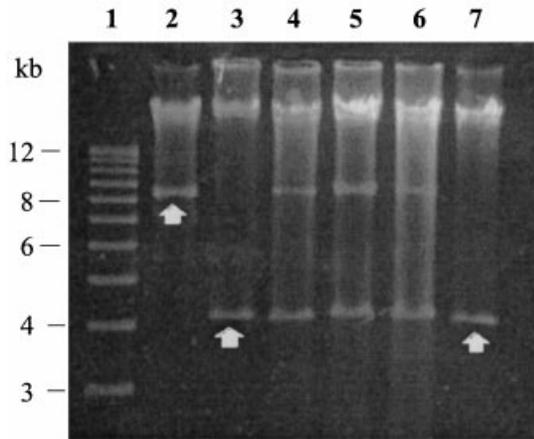
**Nucleotide sequence accession number.** The nucleotide sequence of the *SpliNPV* M2 *NotI* D fragment reported in this paper has been deposited in the databases under accession number AF527603. The predicted protein was analysed using the ANTHEPROT program, version 4.9 (Deléage *et al.*, 1988).

## Results

### Characterization of the *SpliNPV* Δ4 mutant, non-infectious *per os*

While evaluating the *per os* pathogenicity for *S. littoralis* larvae of the OBs of various *SpliNPV* clonal isolates, one appeared to be non-infectious, although it retained its ability to replicate in cell culture. This result led to a more detailed study of this virus mutant. Restriction endonuclease analysis revealed a deletion of 4.5 kb in the non-infectious mutant, designated *SpliNPV* Δ4. This deletion is located in the *NotI* D restriction fragment (Figs 1 and 2, lane 3).

The complete fragment was cloned in both the wild-type and the *SpliNPV* Δ4 mutant to give plasmids p219.1 and p220.32, respectively, and their inserts were sequenced to



**Fig. 2.** Agarose gel electrophoresis of viral DNA restricted by *NotI*. Larvae were infected *per os* with OBs obtained by transfection with SpliNPV M2 (lane 2) or by co-transfection with SpliNPV  $\Delta$ 4 plus p219.1 (lanes 4–6, three different experiments). SpliNPV  $\Delta$ 4 DNA is included as a marker for the *NotI* D fragment (lanes 3 and 7). Lane 1, molecular mass marker X (Boehringer). Arrows indicate the 8.6 or 4.2 kb *NotI* D fragments characteristic of SpliNPV M2 and SpliNPV  $\Delta$ 4, respectively.

determine the differences between both viruses. A 4415 nt long deletion was found and five genes are affected: ORFs 3–7 (Fig. 1A, B). In SpliNPV  $\Delta$ 4, a composite ORF is created by the in-phase fusion of the 5' end of ORF 7 to the 3' end of ORF 3 (Fig. 1B).

Injection of supernatants from SpliNPV  $\Delta$ 4-infected cell culture into larvae led to the death of the larvae from polyhedrosis, similar to that produced by the wild-type virus (Table 1, experiments 1 and 2). The OBs produced by the dead larvae were purified and used for bioassays. Purified SpliNPV  $\Delta$ 4 OBs were still not infectious *per os* (Table 2, compare experiments 1 and 2 with 3 and 4). No difference in morphology was found using scanning electron microscopy between SpliNPV  $\Delta$ 4 and wild-type OBs purified from the dead larvae (data not shown). SpliNPV  $\Delta$ 4 ODVs were able to kill the larvae when injected into the haemocoel but not when the larvae were infected *per os* (Table 1, experiments 4 and 6).

Finally, DNA extracted from ODVs from both viruses was infectious by transfection into Sf52 cells.

### Complementation of the SpliNPV $\Delta$ 4 mutant

The SpliNPV  $\Delta$ 4 polyhedra were not infectious *per os*, therefore a complementation analysis was set up to test which gene(s) could be involved. Purified DNA from SpliNPV  $\Delta$ 4 was co-transfected with p219.1, containing the complete wild-type *NotI* D fragment, which compensates for the deletion. The majority of the larvae ingesting the OBs produced by the co-transfected cells died, confirming that the deletion is responsible for the observed phenotype (Table 3, experiment 5). *NotI* restriction pattern of viral DNA purified from dead larvae always revealed a mixture of wild-type and SpliNPV  $\Delta$ 4 genotypes (Fig. 2, lanes 4–6). Homologous recombination between SpliNPV  $\Delta$ 4 and p219.1 reconstructed a wild-type virus able to infect larvae *per os*. In addition, the presence of SpliNPV  $\Delta$ 4 genotypes indicates that those defective viruses were helped in their passage through the midgut.

When using either p75.36 or p215.4, which span the entire deleted region, no rescue was observed (Table 3, experiments 6 and 7). Only ORF 7 (Fig. 1C) is cut in both plasmids, suggesting that this is the ORF responsible for the observed phenotype. To confirm these results, co-transfections were carried out in a similar way with subclones covering the deletion. Plasmid p228.12 contains only one complete gene affected by the deletion [ORF 3 (*bro-a*), homologous to *Spodoptera litura* NPV (SpltNPV) ORF 120], while p229.41 and p226.1 contain two genes each (ORFs 5 and 6 and ORFs 6 and 7, respectively). ORF 4 (*egt*) was not included because its deletion was shown not to harm polyhedra infectivity either in AcMNPV (O'Reilly & Miller, 1990) or in SpliNPV (G. Croizier, L. Croizier and M. López Ferber, unpublished data).

Larvae died from baculovirus infection only when p226.1 was included in the co-transfection (Table 3, experiment 10). p226.1 presents common sequences with SpliNPV  $\Delta$ 4 only in the 5' portion of ORF 7, the 3' moiety being affected by the deletion (Fig. 1C). Under these conditions, no homologous

**Table 1.** Infectivity of the different baculovirus phenotypes of wild-type SpliNPV (M2 strain) and SpliNPV  $\Delta$ 4 viruses

Experiment	Virus	Virus phenotype	Inoculation method	Larval stage	Total no. of larvae	Dead larvae
1	SpliNPV M2	BV	Injection	L3	48	48
2	SpliNPV $\Delta$ 4	BV	Injection	L3	48	47
3	SpliNPV M2	ODV	Injection	L3	24	24
4	SpliNPV $\Delta$ 4	ODV	Injection	L3	24	21
5	SpliNPV M2	ODV	<i>Per os</i>	Neonates	120	115
6	SpliNPV $\Delta$ 4	ODV	<i>Per os</i>	Neonates	96	1*

\* No OBs observed.

**Table 2.** LD<sub>50</sub> (in OBs per larva) of wild-type SpliNPV (M2 strain) and SpliNPV Δ4 viruses

Experiment	Virus OB	Inoculation method	Larval stage	LD <sub>50</sub>	SE	
1	SpliNPV M2	<i>Per os</i>	Neonates	4.40	0.68	240
2	SpliNPV M2	<i>Per os</i>	L3	35047	5577	120
3	SpliNPV Δ4	<i>Per os</i>	Neonates	No death		240
4	SpliNPV Δ4	<i>Per os</i>	L3	No death		120

**Table 3.** Complementation analysis of the non-infectious polyhedra phenotype

Sl52 cells were infected with virus (experiments 1 and 2), transfected with the viral DNA alone (experiments 3 and 4) or co-transfected with the viral DNA and plasmids or PCR products as indicated. Neonate larvae were fed with the polyhedra produced by the infected cells. At least three independent replicates were done for each experiment. Dead larvae were checked for the presence of OBs.

Experiment	Virus or DNA used to infect the cells		Plasmid	Genes complemented	Total number of larvae	Dead larvae	Rescue
1	Virus	SpliNPV M2			50	50	
2	Virus	SpliNPV Δ4			50	0	
3	DNA	SpliNPV M2			24	24	
4	DNA	SpliNPV Δ4			24	0	
5	DNA	SpliNPV Δ4	p219.1	<i>da26, egt, fgf, ORF 6, ORF 7</i>	24	18–23	+
6	DNA	SpliNPV Δ4	p75.36	<i>da26, egt, fgf, ORF 6</i>	24	0–1*	–
7	DNA	SpliNPV Δ4	p215.4	–	24	0	–
8	DNA	SpliNPV Δ4	p228.12	<i>da26</i>	24	0–2*	–
9	DNA	SpliNPV Δ4	p229.41	<i>fgf, ORF 6</i>	24	0	–
10	DNA	SpliNPV Δ4	p226.1	<i>ORF 6, ORF 7</i>	24	10–14	+
11	DNA	SpliNPV Δ4	PCR 2702	<i>ORF 6, ORF 7</i>	24	9–14	+
12	DNA	SpliNPV Δ4	PCR 1968	<i>ORF 7</i>	24	10–13	+
13	Mock-infected				50	0–2*	

\* Neither polyhedra nor evidence of virus infection was found in the dead larvae.

recombination is possible. The viral DNA contained in the OBs extracted from the dead larvae always presents the 4.2 kb *NotI* D fragment characteristic from SpliNPV Δ4 (data not shown). Larvae fed with those OBs were not killed but ODVs extracted from them were infectious by injection, confirming that the genomes amplified were similar to SpliNPV Δ4 DNA and that no recombination had occurred during the co-transfection.

Plasmid p226.1 encompasses the region between the *SphI* (located at 4520) and the *EcoRI* (7226) sites, including two ORFs. ORF 6 can encode a 77 aa peptide. It is located immediately downstream to ORF 7, which has coding potential for a 525 aa protein.

Although ORF 6 was unlikely to be responsible because p229.41 did not rescue the phenotype (Table 3, experiment 9), a PCR approach was chosen to confirm the involvement of ORF 7 in the observed phenotype.

The promoter region of ORF 7 has not been analysed but the rescue observed in p226.1 suggests that the region upstream of the ORF 7 ATG start codon contained in p226.1

is sufficient to drive transcription. Accordingly, we used the M13 forward primer (FP), thus including the region upstream of ORF 7 containing all of the putative promoter. The second primer, SpliNPVORF7/1, was designed to be complementary to the stop codon of ORF 7, eliminating ORF 6 (Fig. 1C, PCR 1968). A positive control PCR containing the complete insert of p226.1 was obtained by amplifying between the FP and the M13 reverse primer (Fig. 1C, PCR 2702). To eliminate possible read-through artefacts, PCR products were digested at their extremities with *EcoRI/SphI* before being used in co-transfections. To prevent a positive response due to template carry-through, preliminary experiments were set up to reduce the amounts of template in the reactions to obtain negative results. No larva death was recorded when using less than 5 ng of template in a PCR volume of 100 μl.

Co-transfection of cells with SpliNPV Δ4 DNA and PCRs 2702 or 1968 allowed rescue at roughly similar levels to that of p226.1 (Table 3, compare experiments 10, 11 and 12). The rescue observed with PCR 1968 confirms that the presence of

**A**

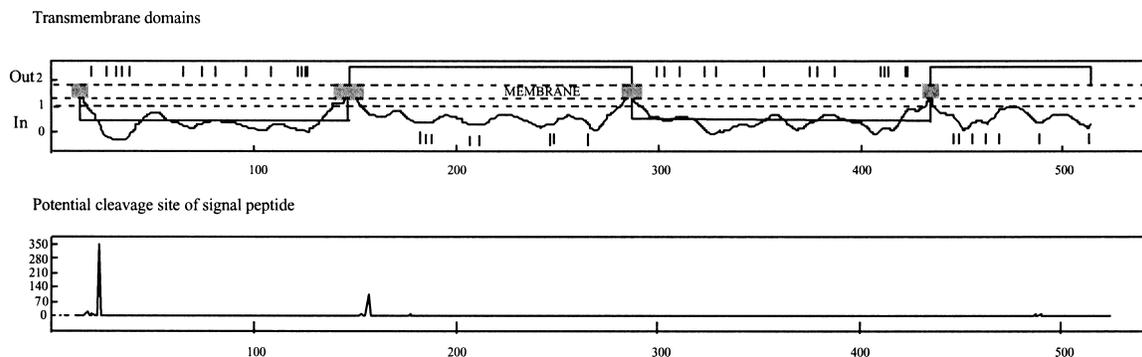
SpliNPV PIF

↓

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rqrarrcnsn tgvwlltesD VGFSLICSCT APGLVTQVNM YEDCDVPVGC LPHGVVADIN 180
EKPIRCKCNS GFVSDFLPNT EIPYCRSQTF RDVLNDTNFV PVAPCPPNYI QVDHPGLPNS 240
YAQYLRNRNA CVPNPCAIDP ITGERHNGII MYDDNTWCG CTSIIGIFFPV Yssggsmlrp 300
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gghdtlyden wsinkfifkf svsytptrvt pgfehiinql tdhaiaynvr qkerplssvc 420
frrpayssct LTFPHITCIL LGSESRFERT CFGVRDTDEI IKMATTAVRY AEYFILTLV 480
SGNFLQSVRP NDNILNTVFG IITLAEEEHG ILRQLLDTYP NYSVR 525
  
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**B**



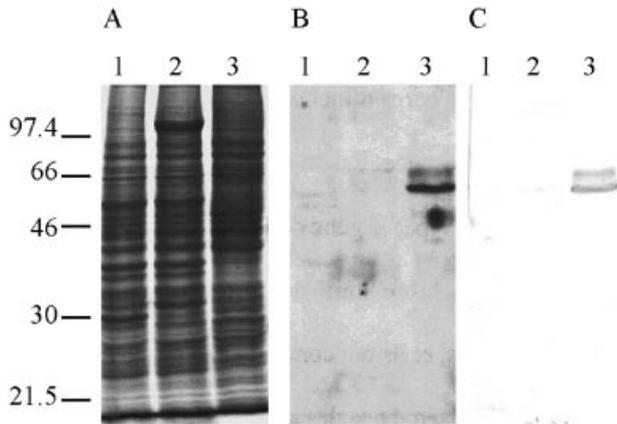
**Fig. 3.** The SpliNPV M2 PIF protein. (A) Amino acid translation of the PIF sequence deduced from the nucleotide sequence. Putative *N*-glycosylation sites are underlined. The residues corresponding to the predicted transmembrane domains (double-underlined), those located inside the cell (lowercase letters) and those predicted to be external to the cell membrane (capital letters) are also indicated. The arrow indicates the potential secretion signal peptide cleavage. (B) Transmembrane domain profiles and potential cleavage sites of the signal peptide (ANTHEPROT software, version 4.9). The bars in the upper panel indicate R and K residues.

ORF 7 is necessary and sufficient to rescue the infectivity of polyhedra in SpliNPV  $\Delta 4$ .

### Protein sequence analysis

ORF 7 can encode a 525 residue protein (Fig. 3A), with a theoretical molecular mass of 59.6 kDa and a predicted isoelectric point of 5.08. This gene product is well conserved among all sequenced baculoviruses. The maximal coding capacity of these ORFs varies between 510 and 540 aa. The global level of homology at the amino acid level between this ORF in SpliNPV and the homologous genes of AcMNPV (ORF 119, Ayres *et al.*, 1994), *Bombyx mori nucleopolyhedrovirus* (BmNPV) (ORF 97, Gomi *et al.*, 1999), *Orgyia pseudotsugata nucleopolyhedrovirus* (OpMNPV) (ORF 119, Ahrens *et al.*, 1997), LdMNPV (ORF 155, Kuzio *et al.*, 1999), *Spodoptera exigua*

*nucleopolyhedrovirus* (ORF 36, IJkel *et al.*, 1999), SpltNPV (ORF 124, Pang *et al.*, 2001), *Helicoverpa armigera* NPV (ORF 111, Chen *et al.*, 2001), *Epiphyas postvittana* NPV (ORF 106, Hyink *et al.*, 2002), *Xestia c-nigrum granulovirus* (ORF 84, Hayakawa *et al.*, 1999), *Plutella xylostella granulovirus* (ORF 7, Hashimoto *et al.*, 2000), *Cydia pomonella granulovirus* (ORF 75, Luque *et al.*, 2001), *Phthorimaea operculella granulovirus* (ORF 66, L. Croizier, A. Taha, G. Croizier and M. López Ferber, unpublished, GenBank accession number AF499596) and *Culex nigripalpus baculovirus* (ORF 29, Afonso *et al.*, 2001) ranges around 40%. In all baculoviruses, 50 aa are absolutely conserved; among them, 19 of the 24 cysteines present in the SpliNPV gene are conserved. The most conserved portion of the protein is between residues 147 and 174, with 10 residues perfectly conserved in all baculoviruses. Higher variation is



**Fig. 4.** Detection of His-tagged SpliNPV PIF expressed in recombinant AcMNPV BacPAKPIFHIS. Total proteins extracted from mock-infected cells (lane 1), cells infected with the parent AcMNPV virus encoding the *lacZ* gene (lane 2) or cells infected with the recombinant virus (lane 3) were separated by electrophoresis. Similar gels were stained (A) or transferred to PVDF membranes (B, C). Western blots were developed using Ab262 and chemiluminescence (B) or AbHis and peroxidase chromogenic reaction (C).

observed in the C-terminal moiety, where it is difficult to find a consensus.

A consensus secretion signal peptide is detected both by the GCG program SPSCAN and the program ANTHEPROT (version 4.9), with values of  $P = 2.02 \times 10^{-4}$  and 350, respectively (Fig. 3B). A second putative cleavage site is detected after residue Q<sup>157</sup> (ANTHEPROT value = 108) (Fig. 3B).

ANTHEPROT predicts four transmembrane domains that delimit two intracellular and two extracellular regions (Fig. 3A, B). The consensus of secondary structure predictions (ANTHEPROT/NPSA) yields 18%  $\alpha$ -helixes, 24% extended strands and 56% random coils. The most antigenic regions were predicted to be aa 163–179 and 429–442.

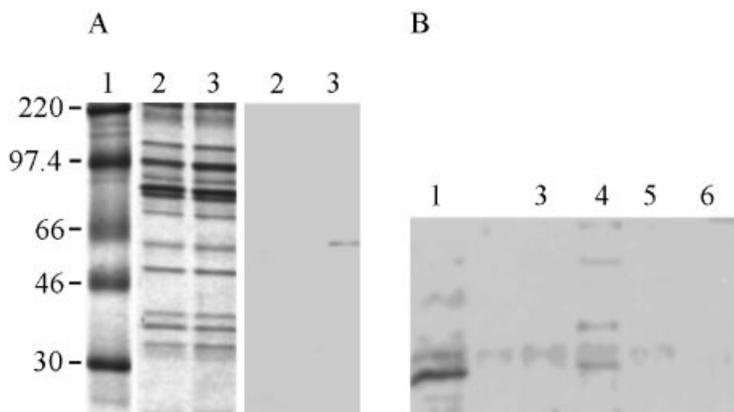
Four putative *N*-glycosylation sites were detected (Fig. 3A). Two of them, <sup>215</sup>NDTN<sup>218</sup> and <sup>521</sup>NYSV<sup>524</sup> are predicted to be located in the external side of the membrane.

### SpliNPV ORF 7 gene product is a structural protein of the ODV

Among the two predicted immunogenic peptides, only the antibody raised against Spli7-1 (Ab262) was sensitive and specific enough to detect a very weak band on Western blots of SpliNPV M2 ODVs. To confirm that this antibody is able to recognize the genuine ORF 7 gene product, the recombinant AcMNPV BacPAKPIFHIS was constructed. Commercial anti-HIS antibodies or Ab262 recognize the same two bands in Western blots of Sf9 cells infected by AcMNPV BacPAKPIFHIS, confirming the specificity of Ab262 (Fig. 4). We have not investigated in detail why two bands are recognized but the upper band probably corresponds to the PIF precursor with uncleaved signal peptide. A single immunoreactive band is observed in SpliNPV M2 but not in SpliNPV  $\Delta 4$  ODVs (Fig. 5A), neither is it observed in the BVs. As a positive control, the recombinant protein produced by AcMNPV BacPAKPIF (Fig. 5A, lane 1) was used. After fractionation of SpliNPV M2 ODV nucleocapsids and envelopes, the immunoreactive protein appears to be linked to the latter (Fig. 5B). The possible glycosylation of the protein has been checked. No sensitivity to F-endoglycosidase (measured by electrophoretic mobility) has been detected, suggesting that none of the potential *N*-glycosylation sites is used. *N*-Glycosylation analysis was supplemented by detection of total glycoproteins using a concanavalin A-binding assay on total ODV proteins. No differences could be observed between the glycosylated protein patterns of SpliNPV M2 and SpliNPV  $\Delta 4$  ODVs (data not shown).

### Discussion

Analysis of SpliNPV  $\Delta 4$ , a deletion mutant isolated in our laboratory, revealed its inability to infect *S. littoralis* larvae *per os*, while retaining the ability to kill upon injection with virus particles. The deletion is contained within the *NotI* D restriction fragment. This fragment was sequenced in both wild-type



**Fig. 5.** Localization of PIF proteins in SpliNPV. (A) SDS-PAGE and Western blot of purified ODVs with Ab262. Lane 1, molecular mass marker; lane 2, deletion mutant SpliNPV  $\Delta 4$ ; lane 3, wild-type SpliNPV clone M2. (B) Western blots on ODV fractions. Lane 1, control BacPAKPIF-infected Sf9 cells; lane 3, SpliNPV  $\Delta 4$  envelopes; lane 4, SpliNPV M2 envelopes; lane 5, SpliNPV  $\Delta 4$  nucleocapsids; lane 6, SpliNPV M2 nucleocapsids.

SpliNPV M2 and SpliNPV  $\Delta 4$ . Compared with the recently published sequence of a SpltNPV (Pang *et al.*, 2001), the SpliNPV M2 *NotI* D fragment lacks ORFs 125–127, both included, which correspond to a second *bro* gene (*bro-b*) and two ORFs with unknown function.

A deletion of 4415 nt was found in SpliNPV  $\Delta 4$  (Fig. 2). This deletion eliminates the *egt*, *fgf* and ORF 6 genes and creates a composite ORF between the N-terminal part of ORF 7 (84 codons) and most of ORF 3 (from codon 46 to the end) (Fig. 1A, B). ORF 3 is homologous to SpltNPV ORF 120 (Pang *et al.*, 2001), which these authors consider as a member of the *bro* gene family (Kuzio *et al.*, 1999). It has been shown that deletion of some *bro* genes impairs virus survival in BmNPV strain T3 (Kang *et al.*, 1999). The number of *bro* genes varies not only between virus species but also between virus strains (López Ferber *et al.*, 2001).

The genes involved in the absence of infectivity of polyhedra are located in the observed deletion, as co-transfection with plasmid p219.1, which covers the deletion, generates fully functional viruses (Table 3, experiment 5). By using a set of plasmids and PCR fragments in co-transfection experiments, it has been possible to attribute the phenotype exclusively to the absence of ORF 7 in the mutant virus.

When co-transfecting SpliNPV  $\Delta 4$  DNA with either plasmid p226.1 or PCR fragments containing the complete ORF 7, no recombinant viruses can be recovered that have integrated the complete ORF 7 by recombination, as there is no sequence homology between the viral DNA and the plasmid at the 3' extremity of the gene. The OBs produced by those co-transfections are infectious *per os* but the virions do not contain the gene. Analysis of both the infectivity of the OBs recovered from the killed larvae and the DNA extracted from them confirmed that the gene has not been inserted.

These results reveal that the product of ORF 7 is not required once the first round of replication has begun, as the progeny viruses do not contain the gene. Accordingly, ORF 7 has been named the *per os* infectivity factor or *pif*.

The N-terminal hydrophobic sequence of PIF is predicted to act as a signal peptide, suggesting that the protein could be either secreted or located in the membrane. The protein is found, however, on the nuclei of the infected cells, in the ODV envelope. The mechanisms involved in nuclear targeting are unknown. Other signal peptide-containing viral proteins have been described with similar localization, like OpMNPV P91 (expression product of OpMNPV ORF 86) (Russell & Rohrmann, 1997), suggesting the existence of a common mechanism.

The main difference between BVs and ODVs is the route of entry into the cells. To start the infection, the OBs ingested by the larva must be dissolved and the virions they contain released into the midgut lumen. A second step is the degradation of the peritrophic membrane to allow the virions to reach the brush-border cells. The last step is the binding to and entry of the virus into the cells, followed by migration of

the nucleocapsids to the nuclei, liberation of the genome, replication and production of progeny viruses, which would be released to infect other cells. As SpliNPV  $\Delta 4$  BVs or ODVs are infectious both to cells in culture and to the larvae by injection, they cannot lack any essential component required once the nucleocapsid reaches the nucleus.

Two different kinds of proteins have been described that act exclusively on the infectivity of OBs: those facilitating the onset of the infection and the P74 homologues.

Proteins belonging to the first category are trapped inside the OBs from where they are released when the OB is dissolved in the midgut but they are not components of the virions. Among these proteins are the synergistic factors (reviewed by Tanada, 1985) and enhancins or VEFs (Derksen & Granados, 1988). These factors were originally found in granuloviruses but, recently, VEF genes have been found in LdMNPV (Kuzio *et al.*, 1999; Popham *et al.*, 2001). It is possible to supplement a virus by adding the VEF to the mixture of OBs (Gallo *et al.*, 1991). Synergistic factors and VEFs disrupt the peritrophic membranes, facilitating the passage of virions to adhere to the midgut columnar cells (Derksen & Granados, 1988). Another function attributed to synergistic factors was the increase of the fusion of nucleocapsids with the brush-border cells (Uchima *et al.*, 1988). Wang *et al.* (1994) observed that the binding activity of the VEF of *Trichoplusia ni granulovirus* was not required for enhancing activity. All VEFs characterized present the consensus HEXXH, a signature of metalloproteases (Lepore *et al.*, 1996; Bischoff & Slavicek, 1997). No such consensus has been found in ORF 7. Curiously, Derksen & Granados (1988) detected a degradation of the peritrophic membrane of *T. ni* larvae following infection by AcMNPV, a virus in which no gene homologous to the known VEFs has been found. Proteins from other viruses have been shown to enhance baculovirus infectivity, like the entomopoxvirus spindle protein (Mitsuhashi *et al.*, 1998; Hukuhara & Wijnarko, 2001), but the mechanisms involved are unclear. This protein presents between 30 and 40% homology with the baculovirus GP37 glycoprotein. GP37 concentrates in the cytoplasm of infected cells (Gross *et al.*, 1993) and is not essential for virus replication (Cheng *et al.*, 2001).

Sucrose gradient-purified SpliNPV M2 ODVs are infectious *per os*, while similarly obtained SpliNPV  $\Delta 4$  ODVs are not (Table 1). In addition, all attempts to rescue the SpliNPV  $\Delta 4$  ODV with the protein fraction of dissolved OBs from wild-type SpliNPV were unsuccessful (data not shown). Finally, PIF appears to be located in the envelope fraction of ODVs. It is thus unlikely that PIF could be involved either in the dissolution of OBs or in the degradation of the peritrophic membrane.

P74 has been described previously by Faulkner *et al.* (1997) in AcMNPV as a structural ODV protein required for infectivity of polyhedra. Deletion or disruption of the AcMNPV *p74* gene results in a complete absence of the *per os* infectivity of OBs, while injection of ODVs into the haemocoel

leads to polyhedrosis. This phenotype is similar to that obtained with SpliNPV  $\Delta 4$ . A homologous gene has been found in SpliNPV (Faktor *et al.*, 1997). P74 and PIF may interact to achieve entry into cells, either by direct contact or by acting in a cascade in the two steps proposed by Horton & Burand (1993).

In summary, it appears that PIF is a protein required between the binding of ODVs to the midgut cells and the beginning of DNA replication of the virus with the subsequent production of progeny BVs. Further experiments would allow the determination of the precise steps in which the deletion mutant SpliNPV  $\Delta 4$  is impaired and the possible interactions with SpliNPV P74.

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