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Virus entry or the primary infection cycle are not the principal determinants of host specificity of *Spodoptera* spp. nucleopolyhedroviruses

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The multicapsid nucleopolyhedroviruses (NPVs) of *Spodoptera exigua* (SeMNPV), *Spodoptera frugiperda* (SfMNPV), and *Spodoptera littoralis* (SpliNPV) are genetically similar (78 % similarity) but differ in their degree of host specificity. Infection by each of the three NPVs in these three *Spodoptera* host species was determined by oral inoculation of larvae with occlusion bodies (OBs) or intrahaemocoelic injection with occlusion derived virions (ODVs). RT-PCR analysis of total RNA from inoculated insects, targeted at immediate early (*ie-0*), early (*egt*, DNA polymerase), late (chitinase) and very late genes (polyhedrin), indicated that each of the NPVs initiated an infection in all three host species tested. SpliMNPV produced a fatal NPV disease in both heterologous hosts, *S. frugiperda* and *S. exigua*, by oral inoculation or injection. SfMNPV was lethal to heterologous hosts, *S. exigua* and *S. littoralis*, but infected larvae did not melt and disintegrate, and progeny OBs were not observed. SeMNPV was able to replicate in heterologous hosts and all genes required for replication were present in the genome, as the virus primary infection cycle was observed. However, gene expression was significantly lower in heterologous hosts. SeMNPV pathogenesis in *S. frugiperda* and *S. littoralis* was blocked at the haemocoel transmission stage and very nearly cleared. SeMNPV mixtures with SpliMNPV or SfMNPV did not extend the host range of SeMNPV; in all cases, only the homologous virus was observed to proliferate. It is concluded that entry and the primary virus infection cycle are not the only, or the major determinants, for SeMNPV infection of heterologous *Spodoptera* species.

INTRODUCTION

One of the most interesting features of nucleopolyhedroviruses (NPVs) (*Nucleopolyhedrovirus*; *Baculoviridae*) as biological control agents is their restricted host range, which is frequently limited to one or a few related insect species (Gröner, 1986). This is ecologically desirable as it limits the impact of NPV-based biopesticides on non-target species. However, it also represents an important commercial drawback, restricting the use of these products to specific key pests or closely related pest complexes, such as *Heliothis* and *Helicoverpa* species (Chakraborty *et al.*, 1999).

Following consumption of occlusion bodies by the host insect and the liberation of occlusion-derived virions in the midgut, the principal steps of the baculovirus infection cycle involve entry into midgut columnar epithelial cells, the expression of viral early genes, DNA replication, late and very late gene expression, the production and release of budded virus, and occlusion body formation (Miller & Lu, 1997). The host range of any virus is determined by its ability to enter the cells of susceptible hosts, and then to replicate and produce new infectious virus particles. Although several mechanisms operate in conferring host cell specificity, little is known about the critical points at which the infection process is blocked in non-productive infection (Martin & Croizier, 1997; Yanase *et al.*, 1998b). Elucidating the factors that are involved in determining the host range of baculoviruses is pertinent to understanding insect–pathogen interactions and the application of baculoviruses as biopesticides.

Combining genomic elements from viruses possessing different host ranges offers a method of producing recombinant viruses with an extended host range (Kondo & Maeda, 1991). Blockage of *Autographa californica* NPV (AcMNPV) replication in *Bombyx mori* cells could be overcome by homologous recombination between the genomic DNA of AcMNPV and a 133 bp fragment of the helicase gene from *B. mori* NPV (BmNPV) (Croizier *et al.*, 1994). Martin & Croizier (1997) investigated the infectivity of BmNPV

in *Spodoptera frugiperda* (Sf) cell lines, non-permissive for BmNPV. They suggested that BmNPV virus progeny particles did not proliferate in cell culture due to a breakdown in cell-to-cell transmission in Sf9 cells.

The fall armyworm, *S. frugiperda*, the beet armyworm, *Spodoptera exigua* and the Egyptian cotton worm, *Spodoptera littoralis*, are polyphagous insects that regularly cause severe damage to a wide variety of crops in many parts of the world (Brown & Delhurst, 1975). The development of NPV-based biopesticides against these species has attracted attention for their potential implementation in integrated pest management programmes (Moscardi, 1999).

The NPVs of *S. frugiperda* (SfMNPV), *S. exigua* (SeMNPV) and *S. littoralis* (SpliNPV) are virulent pathogens of their homologous hosts, but present a very variable response to heterologous hosts. Larvae of *S. frugiperda* and *S. littoralis* are considered non-permissive to SeMNPV, whereas larvae of *S. exigua* and *S. littoralis* are considered semi-permissive to SfMNPV, and all three *Spodoptera* species are permissive to SpliNPV (Murillo *et al.*, 2003). It has been reported that SeMNPV is only capable of productive infection in *S. exigua* cell lines (Yanase *et al.*, 1998b). This virus can initiate replication in non-permissive insect cell lines including *S. frugiperda*, *Spodoptera litura*, *S. littoralis*, *B. mori* and *Trichoplusia ni*, but replication is restricted at various points, depending on the cell line (Yanase *et al.*, 1998b). Moreover, SeMNPV is capable of DNA replication in *S. frugiperda* cells co-infected with SeMNPV and AcMNPV (Yanase *et al.*, 1998a). SeMNPV and SfMNPV are closely related baculoviruses and their genomes present over 78 % identity (Tumilasci *et al.*, 2003). Despite their high degree of similarity, these two viruses have different host ranges. Studies of SeMNPV behaviour in *S. frugiperda* or *S. littoralis* hosts (considered to be non-permissive) or SfMNPV in *S. exigua* and *S. littoralis* hosts (considered to be semi-permissive), therefore represent an intriguing model to investigate the genetic determinants of baculovirus host specificity.

Most studies of baculovirus specificity have been performed in cell culture, particularly those related to SeMNPV. More realistic studies *in vivo* in non-permissive insects can reveal which step of the virus infection cycle is responsible for blocking replication, resulting in a non-productive infection. Is the entry into gut epithelial cells an important barrier for the virus? Or is it at the level of DNA replication or protein synthesis that virus propagation is impeded in heterologous hosts? Particularly for studies on virus entry, it is necessary to examine the behaviour of the virus *in vivo*.

In this study, we report the effects of SeMNPV infection alone or in combination with SfMNPV or SpliNPV in three *Spodoptera* species. We determine the time-course of SeMNPV infection in the midgut (primary infection) and in the haemocoel (secondary infection) of heterologous hosts. We follow the early events of SeMNPV pathogenesis in *S. frugiperda* and *S. littoralis* and show that following primary infection, SeMNPV proliferation was blocked at the haemocoel transmission stage and virtually cleared.

METHODS

Insects and viruses. Larvae of *S. frugiperda*, *S. exigua* and *S. littoralis* were obtained from laboratory colonies maintained at constant temperature (25 °C), humidity (70 %) and photoperiod (16 h light, 8 h dark), and reared on a wheatgerm-based semi-synthetic diet (Greene *et al.*, 1976). The three NPVs used in this study were: (i) a wild-type Nicaraguan isolate of the SfMNPV, hereafter named SfNIC (Escribano *et al.*, 1999); (ii) a SeMNPV wild-type isolate, named SeUS1 (Gelernter & Federici, 1986; Muñoz *et al.*, 1998), received from M. D. Summers (Texas A & M University, College Station, TX); and (iii) a plaque-purified variant of SpliNPV from Morocco, named SpliM2 (Croizier *et al.*, 1989), provided by G. Croizier (INRA, France). The SfMNPV (Simón *et al.*, 2004) and the SeMNPV (Muñoz *et al.*, 1998) isolates consist of various genotypic variants, resulting in the presence of submolar fragments in restriction enzyme analyses (REN).

These viruses were propagated in fourth instars of their respective homologous hosts by the droplet feeding method (Hughes & Wood, 1981; Caballero *et al.*, 1992; Muñoz *et al.*, 1998). Infected larvae were reared on formaldehyde-free diet and collected after death (4–7 days post-inoculation). Occlusion bodies (OBs) were extracted from dead diseased larvae by homogenizing insect corpses in sterile distilled water and filtering through a cheesecloth. OBs were washed twice with 0·1 % SDS and once with 0·1M NaCl and finally resuspended in bidistilled water. OB suspensions were quantified using a bacterial counting chamber and stored at 4 °C until use.

Inoculation of larvae. To determine the infectivity of SfNIC, SeUS1 and SpliM2, larvae of *S. frugiperda*, *S. exigua* and *S. littoralis* were inoculated with all viruses *per os* or by intrahaemocoelic injection. *Per os* bioassays were performed by inoculating 50 newly moulted second instars by the droplet feeding method. Individual viruses (SfNIC, SeUS1 and SpliM2) as well as three mixtures of OBs (SeUS1/SfNIC, SeUS1/SpliM2 and SfNIC/SpliM2 in a ratio of 1 : 1, and SeUS1/SfNIC and SeUS1/SpliM2 in a ratio of 1 : 100 000) were used as inocula. For each virus inoculum, a single high concentration of OBs was used in order to cause high mortality, as observed by Murillo *et al.* (2003). Intrahaemocoelic injection bioassays were performed with 25 newly moulted fourth instars, using the same virus inocula and the appropriate concentration of OBs for fourth instar insects. The OB concentration used was approximately 10^8–10^9 OBs ml^{-1}. Each larvae was injected with 8 μl occlusion derived virions (ODVs) obtained after alkali treatment of the corresponding concentration of OBs (1 : 1 : 5 OBs : 0·5 M Na_2CO_3 : H_2O by volume) (López-Ferber *et al.*, 2003). Inoculated larvae were individually transferred to a 25 compartment Petri dish and provided with diet. Control larvae were treated identically with solutions not containing virus. All procedures were performed at 25 ± 1 °C.

DNA extraction and endonuclease analysis. OBs obtained from inoculated larvae were purified as described above. ODVs extraction from OBs was performed by incubation with SDS and Na_2CO_3 solution. DNA was extracted from ODVs by incubation with SDS and proteinase K, followed by phenol/chloroform extraction and alcohol precipitation (Croizier & Ribeiro, 1992; Muñoz *et al.*, 1998). The DNA concentration was estimated by agarose gel electrophoresis. For REN analysis, 2 μg viral DNA was mixed with 10 units of the restriction enzyme *Pst*I (Amersham) and the mixture was incubated at 37 °C for 4–12 h. Reactions were stopped at 65 °C for 15 min and mixed with 4 μl loading buffer solution (0·25 %, w/v bromophenol blue, 40 %, w/v sucrose). Electrophoresis was performed using

horizontal 1 % agarose gels in TAE (0·04 M Tris/acetate, 0·001 M EDTA, pH 8·0) and the DNA fragments were visualized by staining with ethidium bromide.

RNA extraction from larvae. Total RNA was extracted following the manufacturer's instructions. Whole inoculated larvae used in detection of SeUS1, SfNIC and SpliM2 viral transcripts were triturated in 500 μl Trizol (Invitrogen) (1 ml/100 mg tissue). The suspension was then mixed with 200 μl chloroform, incubated for 10 min and centrifuged at 13 000 ***g*** for 15 min at 4 °C. The aqueous fraction was precipitated using 2-propanol, centrifuged at 13 000 ***g*** for 10 min at 4 °C and washed with 70 % ethanol. Purified RNA was resuspended in approximately 50 μl H_2O, depending on the size of the final pellet observed. RNA solutions were incubated at 60 °C for 10 min to favour resuspension, and in some cases, it was necessary to freeze and thaw the samples several times. RNA was quantified by measuring absorbance at 260 nm in a spectrophotometer (Hitachi, model U-1100) and stored at −80 °C until used. All materials and reagents were previously sterilized and treated with diethyl pyrocarbonate to eliminate RNases.

Total RNA from SeUS1-, SfNIC- and SpliM2-infected *Spodoptera* larvae was extracted at 6, 12 and 24 h post-inoculation (p.i.). Total RNA from the midgut and the haemocoel of mock-infected and SeUS1-infected fourth instar *Spodoptera* larvae was extracted at 24, 48, 72, 120, 144 and 168 h p.i. and from the pupae of *S. frugiperda* and *S. littoralis* as described previously.

Detection of viral transcripts. RT-PCR was performed to detect gene expression in insect larvae and pupae, and to determine the presence or absence of SfNIC, SeUS1 and SpliM2 gene transcripts. After treatment with DNase, equivalent amounts of RNA (0·6 μg) were used in each reaction. To verify the absence of contaminant DNA in the samples, a PCR was performed on all RNA samples. RT-PCR was performed in two different steps. First, cDNA synthesis was performed using the Improm-II reverse transcriptase (Promega) and the internal reverse oligonucleotides specific to the viral genes described in Table 1, according to the manufacturer's instructions. An aliquot of the reaction (1/4) was then subjected to PCR amplification with a *Taq* DNA polymerase (Bioline) and the forward and reverse primer mixture for each gene (Table 1). PCR products were analysed in 1 % agarose gels. A 100 bp marker ladder (Invitrogen), containing fragments of 0·1–2·6 kb in size, was used for size determination. DNA fragments were stained with ethidium bromide, visualized in a UV transilluminator, photographed and examined using the Molecular Analyst program (Bio-Rad).

To determine the capacity of the virus to enter midgut epithelial cells we used specific primers for the immediate early transcribed *ie-0* gene of SfNIC (Simón *et al.*, 2004) and SeUS1 (Ijkel *et al.*, 1999), and the early transcribed *egt* gene of SpliM2 (Faktor *et al.*, 1995) (Table 1). RT-PCR was performed on total RNA extracted from SfNIC-, SeUS1- or SpliM2-infected larvae at 6, 12 and 24 h p.i. Secondly, SeUS1 infection in the three *Spodoptera* host species was followed in detail. The transcription signals of *ie-0*, DNA polymerase (an early gene), chitinase

Table 1. Oligonucleotides used in the study

Primer	Sequence (position in the genome)	Amplification purpose
Sfie0.1	5′-TACGCTCGAGATGAGTATTAATCATGATTC-3′	Forward and reverse primers that amplified 500 bp central fragment of early transcribed SfNIC *ie-0* gene
Sfie0.2	5′-CGTACTCGAGTCTGGCAAATGTTACACT-3′	
Seie0.1	5′-CTATAGCTCGACGCTCGGTG-3′ (131937–131956)	Reverse and forward primers that amplified 510 bp fragment in 3′ extremity of early transcribed SeUS1 *ie-0* gene
Seie0.2	5′-ATCGTCTTCGATACCGCGAG-3′ (132428–132447)	
SeDNApol.1	5′-ATGACTTCTTCGTCGTCGTC-3′ (90226–90245)	Forward and reverse primers that amplified 319 bp fragment in 5′ extremity of early transcribed SfNIC DNA polymerase gene
SeDNApol.2	5′-TAGCACGTCGTGTTAGCGTG-3′ (90526–90545)	
Sechit.1	5′-ATGCCCTATATTATTACGCT-3′ (21122–21141)	Forward and reverse primers that amplified 339 bp fragment in 5′ extremity of late transcribed SfNIC chitinase gene
Sechit.3	5′-AGCAACCGTCGACGTTGCAT-3′ (21442–21461)	
Sepolh.1	5′-ATGTATACTCGCTACAGCTA-3′ (1–20)	Forward and reverse primers that amplified 319 bp fragment in 5′ extremity of very late transcribed SfNIC polyhedrin gene
Sepolh.2	5′-TGTCTTCCATGAAACGCGTC-3′ (300–319)	
Spliegt.1	5′-TAGTAATGAGCATCGGAAGA-3′	Reverse and forward primers that amplified 370 bp fragment in 3′ extremity of early transcribed SpliM2 *egt*
Spliegt.2	5′-TCGACAGACGAAGCCATAGA-3′	

(late gene) and polyhedrin gene (very late gene) were analysed by RT-PCR using total RNA extracted from the three host species infected *per os* and by injection with SeUS1 at 24 and 48 h p.i. Finally, to determine whether SeUS1 was able to produce secondary infection, RT-PCR was performed using total RNA extracted from midgut cells and the haemocoel of infected *Spodoptera* larvae at 24, 48, 72, 120, 144 and 168 h p.i., as described above, and from pupae of *S. frugiperda* and *S. littoralis* larvae (all *S. exigua* larvae died prior to pupation), using two internal primers to the SeUS1-polyhedrin gene (Table 1).

Detection of SfNIC, SeUS1 and SpliM2 DNA in infected and co-infected larvae. The presence of viral DNA was investigated by PCR (Sambrook *et al.*, 1989) with total DNA extracted as described previously. Volumes of 0·1 μl of the DNA solution were used for each reaction (approx. 10 ng). PCR was performed to detect SfNIC, SeUS1 and/or SpliM2 DNA in larvae inoculated with SfNIC, SeUS1 or SpliM2, or with a virus mixture (SfNIC/SeUS1, SeUS1/SpliM2 or SfNIC/SpliM2) as described previously. Infected larvae were sampled immediately following death (5–6 days) in the case of *S. exigua* and at the same moment for the other species, whether or not they had died of polyhedrosis disease. Specific primers were used for the SfNIC and SeUS1 *ie-0* genes, and the SpliM2 *egt* gene (Table 1). PCR products were analysed in 1 % agarose gels and visualized with ethidium bromide as described above.

Quantification of NPV transcripts and genomic DNA. The detection of viral transcripts was performed by RT-PCR using RNA obtained at various intervals post-infection, as described above. For the semi-quantitative detection of viral genomic DNA, a PCR was performed using template DNA obtained from infected larvae at 5–6 days p.i. or co-infected larvae. The RT-PCR and PCR products were analysed in 1 % agarose gels, visualized with ethidium bromide and digitally photographed at a resolution of 19·7 pixels cm^{-1}. The relative intensities of the RT-PCR and PCR products were estimated by densitometric analysis, using the Scion Image PC program (Scion Corporation, USA). The results of densitometric analyses are presented graphically.

RESULTS

Infectivity of SfNIC, SeUS1 and SpliM2 in *Spodoptera* species

The infectivity of each of the three NPVs for the three *Spodoptera* species was determined following oral inoculation of second instars with OBs or injection of fourth instars with ODVs. SpliM2 produced a fatal infection in its homologous host, *S. littoralis*, as well as in the heterologous hosts *S. frugiperda* and *S. exigua* by oral inoculation or by injection of ODVs. In all cases, the infected larvae showed the typical signs and symptoms of NPV disease and DNA extracted from OBs harvested from dead insects showed the typical REN profiles of SpliM2.

SfNIC also produced a fatal infection in all three *Spodoptera* species following consumption or injection of inocula. In *S. frugiperda* the infection produced the typical signs and symptoms of an NPV disease, whereas in *S. littoralis* and *S. exigua* the symptoms were atypical. For example, a very low number of OBs were produced per larva and the infected larvae did not liquefy at the end of the infection process in these hosts. The low number of OBs extracted from infected larvae of heterologous hosts made it impossible to examine the viral DNA profile by REN analysis. In contrast, while SeUS1 produced a fatal infection in its homologous host, *S. exigua*, the larvae of *S. frugiperda* and *S. littoralis* were resistant to SeUS1. Heterologous larvae remained healthy, even after oral inoculation with a high concentration of OBs or injection of a high concentration of ODVs into the haemocoel. No OBs of SeMNPV were visualized in the haemocoel of inoculated heterologous hosts by optical microscopy.

Detection of NPV-specific transcripts in *Spodoptera* larvae

RT-PCR analysis of total RNA extracted from virus-inoculated larvae showed that each of the three NPVs (SfNIC, SeUS1 and SpliM2) initiated an infection in all three host species tested (*S. frugiperda*, *S. exigua* and *S. littoralis*). Following oral inoculation with OBs, SfNIC-*ie-0* or SpliM2-*egt* transcripts were detected at 12 and 24 h p.i. at a similar level in all three host species (Fig. 1a and b). SeUS1-*ie-0* transcripts were also detected in *S. exigua* at 6 h p.i., *S. frugiperda* at 24 h p.i. and *S. littoralis* at 12 h p.i., signalling the onset of infection by SeUS1 in each host species (Fig. 1c). However, in *S. exigua* the transcription level increased between 6 and 24 h p.i., whereas in *S. frugiperda* and *S. littoralis* the transcription of this gene was delayed

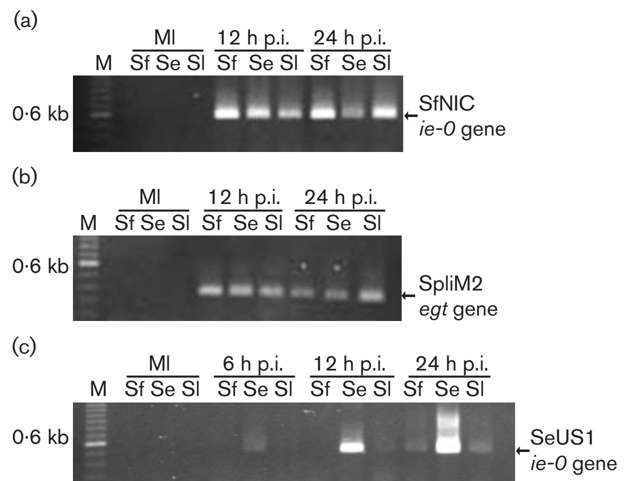

Fig. 1. Detection of early transcripts of SfNIC, SeUS1 and SpliM2 NPVs in *S. frugiperda*, *S. exigua* and *S. littoralis* larvae by RT-PCR. The position of specific RT-PCR products is indicated on the right, the molecular marker used was 100 bp ladder (Invitrogen). (a) RT-PCR analysis of SfNIC *ie-0* gene performed on total RNA extracted from *S. frugiperda* (Sf), *S. exigua* (Se) and *S. littoralis* (Sl) mock-infected (MI) and *per os*-infected with SfNIC at 12 and 24 h p.i. (b) RT-PCR analysis of SpliM2 *egt* gene under the same conditions. (c) RT-PCR analysis of SeUS1 *ie-0* gene performed on total RNA extracted from *S. frugiperda* (Sf), *S. exigua* (Se) and *S. littoralis* (Sl) mock-infected (MI) and *per os*-infected with SeUS1 at 6, 12 and 24 h p.i.

and the quantity of transcript detected by RT-PCR was markedly lower (Fig. 1c).

Further RT-PCR experiments were performed in order to determine if the cascade of gene expression in SeUS1 was blocked at any point in *S. frugiperda* and *S. littoralis*. For this, the transcription of an early (DNA polymerase), a late (chitinase) and a very late (polyhedrin) gene were studied during SeUS1 replication in larvae inoculated orally or by intrahaemocoelic injection. RT-PCR analysis indicated the presence of NPV-specific transcripts of these three genes in both heterologous hosts and using both methods of inoculation (Fig. 2). The transcriptional activity of DNA polymerase and chitinase genes was very low in comparison with that of the polyhedrin (*polh*) gene (Fig. 2). By 24 and 48 h p.i. the SeUS1-*polh* transcription level was slightly higher in larvae inoculated *per os* than in larvae inoculated by intrahaemocoelic injection, although these differences could be due to the inoculation method used (Fig. 2b). As expected, the transcriptional activity of the three genes studied was markedly higher in *S. exigua* than in the heterologous hosts (Fig. 2).

SeUS1 secondary infection in heterologous hosts

The levels of SeUS1 transcription detected in *S. frugiperda* and *S. littoralis* were more robust than expected, leading us to examine the temporal progression of primary and secondary infection in these hosts compared to *S. exigua*. SeUS1-*polh* transcription was examined from 24 to 168 h p.i. in the intact midgut or the haemocoel of larvae, as well as in pupae. SeUS1-*polh* transcripts were detected at 24 h p.i. in the midgut of the three *Spodoptera* species tested, indicating the occurrence of a primary infection in each case (Fig. 3). In *S. exigua*, the level of SeUS1-*polh* transcription was very high at 24 h p.i. and increased until 120 h p.i. In *S. frugiperda* and *S. littoralis*, the midgut transcriptional activity of SeUS1-*polh* also increased between 24 and 72 h p.i., but was 2·0 and 1·5 times lower, respectively, than in *S. exigua* at 72 h p.i. Transcriptional activity declined markedly in *S. frugiperda* and *S. littoralis* larvae at 120 h p.i. At this time, the transcriptional activity in *S. frugiperda* and *S. littoralis* was 48·3 and 7·2 times lower than in *S. exigua* larvae. Finally, at 168 h p.i., transcription of the *polh* gene was almost undetectable in heterologous hosts, whereas all homologous hosts had died of polyhedrosis in the interval 120–144 h p.i. (Fig. 3b).

SeUS1-*polh* transcripts were also detected in the haemocoel of *S. littoralis* and *S. frugiperda*, signalling the onset of the secondary infection by SeUS1 in these hosts (Fig. 3). In *S. exigua* larvae, the level of transcriptional activity increased markedly between 24 and 120 h p.i., whereas in the heterologous hosts, the transcription of SeUS1-*polh* was much lower. In *S. frugiperda* larvae, the prevalence of *polh* transcripts increased 2·9 times between 24 and 72 h p.i., although the maximum activity observed at 72 h p.i. was 3·7 times lower than in *S. exigua* larvae. After 72 h p.i., the quantity of *polh* transcripts markedly declined, such that by 168 h p.i., the haemocoel infection was almost cleared in the final instar of *S. frugiperda* (Fig. 3b). In *S. littoralis* larvae, the quantity of *polh* transcripts increased 5·2 times between 24 and 120 h p.i., although this was 3·5 times less than observed in *S. exigua* larvae at the same timepoint (120 h p.i.). By 144 and 168 h p.i., the quantity of *polh* transcripts had decreased 6·3- and 18·4-fold, respectively, compared to the maximum observed at 120 h p.i.

Viral progeny in single and double virus-infected larvae

The effect of the homologous NPV on the replication of heterologous NPVs was assessed. Viral DNA produced in *S. exigua*, *S. frugiperda* and *S. littoralis* larvae infected by single NPVs, used as controls, or co-infected with combinations of SeUS1, SfNIC and SpliM2, was analysed by PCR (Fig. 4). The quantity of SeUS1 DNA detected by PCR in larvae of *S. frugiperda* or *S. littoralis* co-infected with SeUS1/SfNIC or SeUS1/SpliM2 was 3·1 and 3·0 times greater, respectively, by *per os* inoculation (Fig. 4a) and 1·6 and 2·2 times greater by intrahaemocoelic inoculation (Fig. 4b), compared to larvae inoculated with SeUS1 alone. However, the reverse was not true; we did not observe a clear increase in the quantity of SfNIC or SpliM2 DNA in heterologous larvae when co-infected with the homologous viruses, as observed

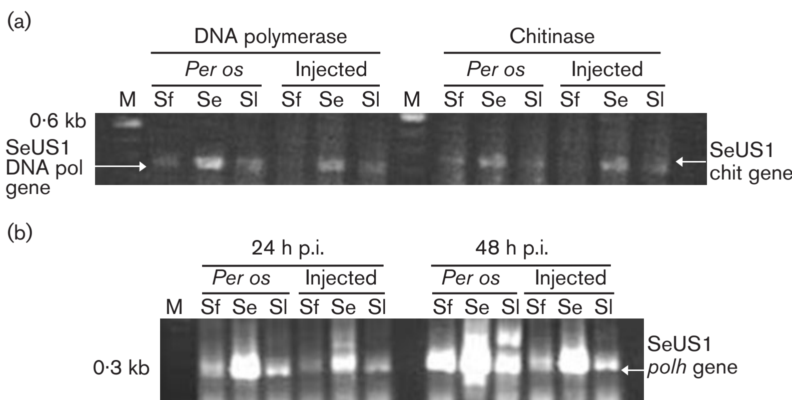

Fig. 2. Detection of the early and late transcribed SeUS1 DNA polymerase and chitinase genes at 48 h p.i. (a), and very late transcribed SeUS1 polyhedrin gene (b) at 24 and 48 h p.i. by RT-PCR on total RNA extracted from *S. frugiperda* (Sf), *S. exigua* (Se) and *S. littoralis* (Sl) larvae inoculated with SeUS1 *per os* and by intrahaemocoelic injection. Specific RT-PCR products are indicated, the molecular marker used was 100 bp ladder (Invitrogen).

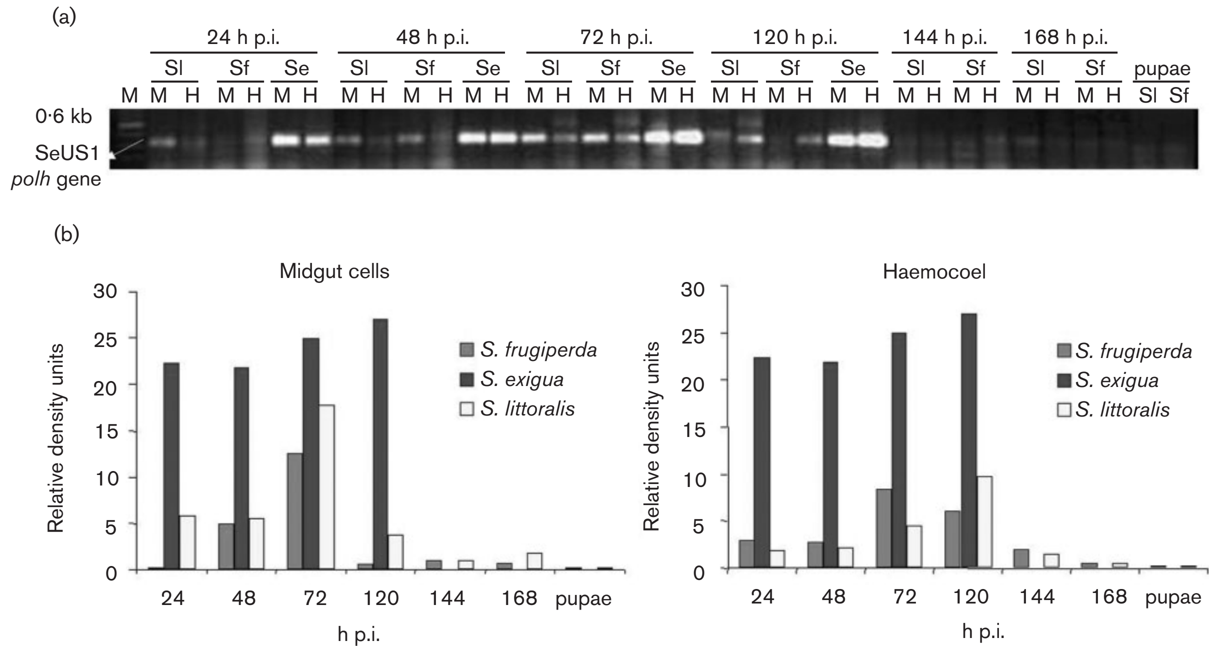

Fig. 3. (a) Detection of SeUS1 secondary infection in heterologous hosts. Detection of SeUS1 polyhedrin gene transcription in midgut (M) and haemolymph (H) of *S. littoralis* (Sl), *S. frugiperda* (Sf) and *S. exigua* (Se) larvae at 24–168 h p.i. and from pupae of heterologous hosts, by RT-PCR using specific primers for the polyhedrin gene of SeUS1. Specific fragments are indicated, the marker used for size determination was 100 bp ladder (Roche). (b) Graphical representation indicating the quantity of RT-PCR products estimated by densitometric analysis.

for SeUS1. In *S. exigua* larvae, the quantity of SfNIC DNA was 1·8 and 1·2 times lower by *per os* and intrahaemocoelic inoculation, respectively, compared to *S. exigua* larvae inoculated with SfNIC alone (Fig. 4a and b). In contrast, in *S. littoralis* larvae double-infected with SfNIC/SpliM2, the quantity of SfNIC DNA was 2·1 times lower by *per os*

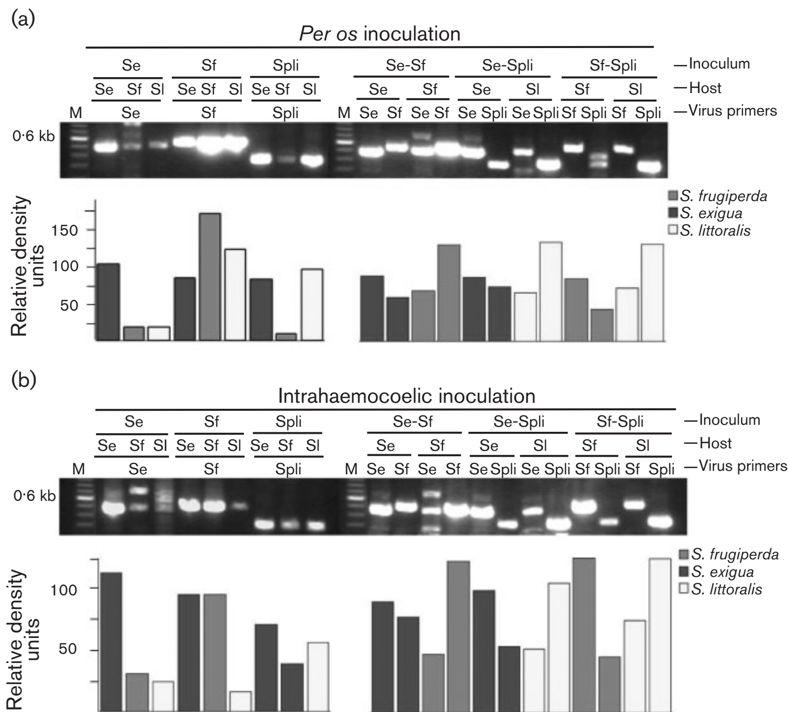

Fig. 4. Detection of NPV DNA by PCR, with specific primers for SeUS1 (Se) and SfNIC (Sf) *ie-0* genes and SpliM2 *egt* (Spli), in *S. littoralis* (Sl), *S. frugiperda* (Sf) and *S. exigua* (Se) larvae after (a) *per os* inoculation or (b) intrahaemocoelic injection of single NPV SeUS1 (Se), SfNIC (Sf) or SpliM2 (Spli), or with co-infections of SeUS1/SfNIC (Se/Sf), SeUS1/SpliM2 (Se/Spli) or SfNIC/SpliM2 (Sf/Spli). A 1 kb ladder (Stratagene) was used for DNA size determination (lane M). Graphical representation below each gel indicates the relative quantities of PCR products estimated by densitometric analysis.

inoculation (Fig. 4a), but 4·7 times greater by intrahaemocoelic inoculation (Fig. 4b), compared to *S. littoralis* inoculated via each route with SfNIC alone. In *S. exigua* larvae co-infected with SpliM2/SeUS1 by both routes, the abundance of SpliM2 DNA decreased slightly ($\sim 1{\cdot}3$ times), compared to the abundance of SpliM2 DNA in *S. exigua* larvae inoculated with SpliM2 alone (Fig. 4a and b). In *S. frugiperda* co-infected orally with SpliM2/SfNIC the abundance of SpliM2 DNA was 3·6 times greater than in *S. frugiperda* larvae inoculated with SpliM2 alone, but did not change in larvae inoculated by intrahaemocoelic injection (Fig. 4b). In general, DNA replication of SeUS1 in heterologous hosts, such as *S. frugiperda* and *S. littoralis*, increased when co-infected with the homologous viruses. Conversely, the DNA replication of wide-host-range viruses in heterologous hosts competed with homologous viruses, resulting in either an increase or a decrease, depending on the hosts and the inoculation route.

To investigate this effect, viral DNA produced in larvae infected by single viruses or larvae co-infected with combinations of SeUS1, SfNIC and SpliM2 was analysed with *Pst*I (data not shown). Viral DNAs extracted from larvae inoculated with each virus singly (SeUS1, SfNIC or SpliM2) consistently showed the typical REN profiles of the virus used as inoculum in all those host species that allowed a productive virus infection. REN profiles of the DNA extracted from OBs harvested from *S. exigua* larvae co-infected with SeUS1/SfNIC or SeUS1/SpliM2 only showed the characteristic restriction fragments corresponding to SeUS1. A similar pattern was observed in *S. frugiperda* larvae co-infected with SeUS1/SfNIC or SfNIC/SpliM2 and in *S. littoralis* larvae co-infected with SeUS1/SpliM2 or SfNIC/SpliM2. In both cases, only OBs corresponding to the SfNIC and the SpliM2 viruses, respectively, were produced in an amount detectable by REN analysis. A similar pattern was observed in larvae infected by intrahaemocoelic injection (data not shown).

In the second inoculation, *S. exigua*, *S. frugiperda* or *S. littoralis* larvae were inoculated with OBs of SfNIC/SeUS1 or SpliM2/SeUS1 at a ratio of 1 : 100 000. A REN analysis with *Pst*I was performed with the DNA from the OBs obtained after inoculation of each host species. We observed similar REN patterns to those observed with 1 : 1 inoculation in all cases (data not shown); we could not detect the SeUS1 virus in co-infected larvae despite its being inoculated in a quantity 10^5 times greater than that of the homologous virus. When interpreting these results, it should be remembered that RT-PCR studies were performed on larval tissues or haemolymph at 48 h p.i., whereas DNA for REN analysis was extracted from OBs harvested from dead larvae.

DISCUSSION

The biological activity and host specificity studies of three *Spodoptera* NPVs revealed differences in their capacity to infect and their pathogenicity in the three *Spodoptera* species tested. SeUS1 caused lethal infections only in its homologous host, *S. exigua*, supporting the current notion that *S. frugiperda* and *S. littoralis* are non-permissive hosts for SeMNPV (Murillo *et al.*, 2003). SpliM2 caused lethal disease in all three host species, whereas SfNIC produced atypical symptoms of infection in the heterologous hosts with no signs of liquefaction or melanization of the cuticle. SfNIC or SpliM2 were capable of DNA replication, gene expression and production of infectious OBs in *Spodoptera* larvae. SeUS1 and SfNIC present high similarity at the genome level (Tumilasci *et al.*, 2003), and probably share a common ancestor. We then asked the question, if SeUS1 is genetically related to SfNIC, why could SeUS1 only cause fatal infections in *S. exigua* and not in *S. frugiperda*? To try to answer this we examined early and late gene expression and DNA replication *in vivo* following infection by SeUS1, SfNIC or SpliM2 alone, or in combination, in homologous and heterologous hosts (Table 2).

We present evidence that all three *Spodoptera* NPVs are able to enter the midgut epithelial cells of the three *Spodoptera* species. Transcription of immediate early genes in SfNIC and SpliM2 was similar in all three host species, indicating that entry into epithelial cells is not a determinant for host range in these viruses. The abundance of SeUS1 transcript was significantly lower in the heterologous hosts, suggesting a reduced level of replication, but clearly SeUS1 virus was able to enter the epithelial cells of these *Spodoptera* species. As transcription of SeUS1-*polh* and DNA replication in the *per os* infections was slightly higher than that observed in infections originating from injected ODVs in all SeUS1-infected host species, we assume that entry into midgut epithelial cells is not the only or the major determinant of SeUS1 host specificity. These results indicate that entry into midgut cells and the primary replication of the viruses do not appear to be the only or the most important restriction to successful infection.

The regulation of 100 or more open reading frames is required to accomplish productive infection by SeMNPV; this is highly complex and involves sequential and coordinated expression of immediately early, early, late and very late genes (Lu & Miller, 1997). In the cascade of gene expression, successive stages of virus replication are dependent on the correct expression of genes from the preceding stages. The transcription of four temporally distinct SeUS1 genes was studied in order to determine the moment at which the virus cycle is blocked. The kinetics of SeUS1 transcription in the three *Spodoptera* species observed by RT-PCR indicated that the virus was actively attempting to replicate in heterologous hosts, although at a much lower level than in its homologous host. The positive replication signal obtained with the late genes chitinase and polyhedrin indicated that all genes required for virus replication in heterologous hosts (Kool *et al.*, 1994) were present in the SeUS1 genome. It is likely that the genes responsible for SeUS1 replication are homologous with those present in SfNIC or SpliM2, since SfNIC and SeUS1 are closely related. The low level of SeUS1

Table 2. Summary of events in the three *Spodoptera* spp. after single infections or co-infections with SeMNPV (Se) and/or SfMNPV (Sf) and/or SpliNPV (Spli) in 1 : 1 mixture or in a ratio of 1 : 100 000

ND, Not determined. In early gene expression + and − indicate the presence or absence of early gene expression or entry into epithelial cells. In DNA replication + and − indicate the presence or absence of detectable DNA by agarose gel electrophoresis or by PCR. In very late gene expression + and − indicate the presence or absence of polyhedrin gene transcription detected by RT-PCR. +++, Abundant; ++, medium; +, low; −, not detectable with the methods used.

Event	Propagating host	Se	Sf	Spli	Se+Sf		Se+Spli		Sf+Spli	
					Se	Sf	Se	Spli	Sf	Spli
Early gene expression										
	S. exigua	+++	+++	+++	ND	ND	ND	ND	ND	ND
	S. frugiperda	+	+++	+++	ND	ND	ND	ND	ND	ND
	S. littoralis		+++	+++	ND	ND	ND	ND	ND	ND
Viral DNA replication										
Detected by gel staining	*S. exigua*	+++	+	+++	+++	−	+++	−	ND	ND
	S. frugiperda	−	+++	+++	−	+++	ND	ND	+++	−
	S. littoralis	−	+	+++	ND	ND	−	+++	−	+++
Detected by PCR	*S. exigua*	+++	++	+++	+++	+	+++	++	ND	ND
	S. frugiperda	+	+++	+++	+	+++	ND	ND	+++	++
	S. littoralis	+	++	+++	ND	ND	++	+++	++	+++
Very late gene expression										
	S. exigua	+++	+++	+++	ND	ND	ND	ND	ND	ND
	S. frugiperda	+	+++	+++	ND	ND	ND	ND	ND	ND
	S. littoralis	+	+++	+++	ND	ND	ND	ND	ND	ND

replication activity in heterologous hosts, as determined by the relatively low levels of SeUS1 gene transcription, could be due to a small number of infected cells producing high levels of virus transcripts, rather than a large number of cells producing very low levels, as has been suggested for BmNPV in Sf9 cell lines (Martin & Croizier, 1997). An alternative hypothesis to account for the low level of SeUS1 gene expression observed in heterologous hosts is that the viral particles budding from infected cells may be unable to infect neighbouring cells, as observed with cells infected with *gp64*$^{-}$ *Autographa californica* multicapsid NPV (AcMNPV) (Monsma *et al.*, 1996). The occurrence of DNA replication and the appearance of an increasing quantity of *polh* transcripts over time are consistent with *S. frugiperda* and *S. littoralis* being semi-permissive species for SeUS1 replication. The lower transcriptional activity of SeUS1 in heterologous hosts could explain why *S. frugiperda* and *S. littoralis* have so far been considered as non-permissive species for SeMNPV.

A further experiment was performed to determine whether SeUS1 is able to produce secondary infections in heterologous hosts. Very late gene expression was studied in the midgut and haemolymph of SeUS1-infected *Spodoptera* larvae. SeUS1 *polh* gene expression was detected in the midgut and the rest of the body indicating that SeUS1 can transmit BVs to neighbouring cells and produce secondary infection in heterologous hosts. In addition, when *S. frugiperda* or *S. littoralis* were each co-infected with SeUS1 and their respective homologous virus, DNA replication of SeUS1 increased with respect to infections of SeUS1 alone. In contrast, by REN analysis we could not observe any characteristic bands of a secondary (heterologous) virus in co-infected larvae, suggesting that REN analysis was probably not sufficiently sensitive to detect low levels of replication in heterologous hosts. We did, however, observe the presence of numerous other bands at low concentrations, probably due to the use of wild-type isolates (SeUS1 and SfNIC) that comprised various genotypic variants. Alternatively, the heterologous infection did not reach the OB production stage. Experiments are in process using pure genotypes in order to detect possible recombination between viruses or genotypic mixtures. However, PCR analysis performed with co-infected larvae indicated that non-permissive virus DNA replication, such as SeUS1, in heterologous hosts (*S. frugiperda* and *S. littoralis*), increased when co-infected with the respective homologous viruses. This suggests that, SfNIC and SpliM2 assist the replication of SeUS1. The origin of DNA replication in SeMNPV, the non-homologous region (*hr*), replicates in AcMNPV co-infected cells at low levels (Heldens *et al.*, 1997), a phenomenon that may have also occurred in *S. frugiperda* and *S. littoralis* larvae. Kamita *et al.* (2003) reached similar conclusions after observing high-frequency recombination between two types of BmNPV in Sf9 cells, a weakly permissive insect cell line, higher than that observed between BmNPV and AcMNPV. High frequencies of recombination indicated that the replication of BmNPV DNAs occurred actively in this cell line (Kamita *et al.*, 2003). After demonstrating that the replication of SeMNPV in *S. frugiperda* cells was improved

by co-infection with AcMNPV, Yanase *et al.* (1998a) suggested that SeMNPV may use the transcripts of AcMNPV for replication in co-infected cells.

We conclude that SeMNPV replication in heterologous hosts requires certain helper functions from SfMNPV or SpliNPV, presumably by sharing some viral factors. Other examples of recombination between viruses or helper functions have demonstrated an increase in DNA replication or the extension of a virus host range. A recombinant AcMNPV bearing a small region of the *p143* gene from BmNPV replicated in a BmN cell line, whereas the original AcMNPV was incapable of replication in these cells (Maeda *et al.*, 1993; Croizier *et al.*, 1994). The replacement or modification of certain NPV genes with those of other NPVs resulted in an extended host range or improved DNA replication, suggesting that these domains might interact with host-specific factors. Other genes that influence baculovirus host range include late transcription factors and apoptotic suppressors. AcMNPV was capable of productive infection in a *Lymantria dispar* (Ld) cell line in the presence of the *hrf-1* gene product from LdMNPV (Thiem *et al.*, 1996; Thiem, 1997). Recently Zhang *et al.* (2002) demonstrated that a host apoptotic response to virus infection reduced AcMNPV cell-to-cell transmission of infection in *S. litura* larvae, apoptosis representing a host-range limiting factor for AcMNPV infection. The molecular mechanisms involved in apoptosis signalling are still unknown. Potential stimuli consist of shut-off of RNA synthesis, viral DNA replication and viral gene expression (Clem & Miller, 1993; Prikhod'ko & Miller, 1996; Miller, 1997; Clem, 2001), and it is possible that several factors are involved in triggering programmed cell death (LaCount & Friesen, 1997).

In the present study, time-course experiments revealed that the quantity of *polh* transcripts in the midgut or haemocoel of SeUS1-infected heterologous hosts decreased significantly at 72–120 h p.i. and subsequently almost disappeared at 168 h p.i., whereas a progressive increase was seen in the homologous host. None of the heterologous larvae succumbed to polyhedrosis disease. Similar results were observed when non-permissive hosts, *Manduca sexta* and *Helicoverpa zea* were inoculated with an AcMNPV recombinant virus expressing the *lacZ* gene (Washburn *et al.*, 1996, 2000). These authors suggested that a cellular immune response was responsible for clearance of a potentially fatal infection of AcMNPV in non-permissive hosts. In both species, primary infection of midgut columnar cells by AcMNPV began at the same time as in permissive hosts, and secondary infections in midgut-associated tracheae were revealed by optical microscope observation of *lacZ* expression. However, in heterologous hosts, a decline in the number of infection foci of AcMNPV was detected. By 72 h p.i., haemocytes surround infected cells and haemocyte aggregations transformed infected cells into melanized capsules. These infections failed to spread and were ultimately cleared. The time-course of SeUS1 infection in heterologous *Spodoptera* hosts is similar to that described by Washburn *et al.* (1996, 2000), although the reporter gene technique they used was far less sensitive than the RT-PCR method that we used. Between 24 and 72 h p.i. SeUS1-*polh* transcription increased but after 72 h p.i. the infection declined and disappeared. It appears that *S. frugiperda* and *S. littoralis* larvae exhibit an immune response to SeUS1 infection. However, due to the technique of midgut dissection we employed, we cannot exclude the possibility that some of the cells intimately associated with the insect midgut, such as tracheal cells, may also have contributed to the results observed in RT-PCR analysis of viral transcripts from midgut tissue.

In conclusion, SpliM2 is able to infect, replicate and produce progeny OBs in all the *Spodoptera* species tested. In contrast, SfNIC is lethal to heterologous hosts *S. exigua* and *S. littoralis* but infected larvae do not melt, liquefy and melanize. The determination of the factors or mechanisms that induce such responses in heterologous hosts infected by SfNIC was not analysed. We could not determine the factors or mechanisms that induce such responses in heterologous hosts infected by SfNIC. Finally, SeUS1 is able to replicate in heterologous hosts and, in addition, all genes required for SeUS1 replication are present in the SeUS1 genome, as the virus infection cycle was observed. However, gene expression is significantly lower in heterologous hosts. It seems that anti-viral responses (apoptosis or cellular immune response) of the heterologous hosts appear to play an important role in the specificity of SeUS1. However, SeUS1 was not blocked at an obvious point during the infection cycle, but declined gradually over time and eventually disappeared. We therefore conclude that entry and the primary virus infection cycle are not the principal determinants for SeUS1 infection of heterologous *Spodoptera* species. Experiments are in progress to determine the mechanisms involved in SeUS1 specificity and which step(s) of the virus cycle are inhibited in heterologous hosts. The system described in this study, SeMNPV, SfMNPV and SpliNPV and their respective hosts, represents a useful model for studying the determinants of baculovirus host range. Such studies can also provide a basis for host-range risk assessment applied to the development of natural and recombinant baculovirus bioinsecticides.

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REFERENCES

Brown, S. E. & Delhurst, C. F. (1975). The genus *Spodoptera* (Lepidoptera: Noctuidae) in Africa and the Near East. *Bull Entomol Res* **65**, 221–262.

Caballero, P., Zuidema, D., Santiago-Alvarez, C. & Vlak, J. M. (1992). Biochemical and biological characterization of four isolates of *Spodoptera exigua* nuclear polyhedrosis virus. *Biocontrol Sci Technol* **2**, 145–157.

Chakraborty, S., Monsour, C., Teakle, R. & Reid, S. (1999). Yield, biological activity, and field performance of a wild-type *Helicoverpa* nucleopolyhedrovirus produced in *H. zea* cell cultures. *J Invertebr Pathol* **73**, 199–205.

Clem, R. J. (2001). Baculoviruses and apoptosis: the good, the bad, and the ugly. *Cell Death Differ* **8**, 137–143.

Clem, R. J. & Miller, L. K. (1993). Apoptosis reduces both the *in vitro* replication and the *in vivo* infectivity of a baculovirus. *J Virol* **67**, 3730–3738.

Croizier, G. & Ribeiro, H. C. T. (1992). Recombination as a possible major cause of genetic heterogeneity in *Anticarsia gemmatalis* nuclear polyhedrosis virus wild populations. *Virus Res* **26**, 183–196.

Croizier, G., Boukhoudmi-Amiri, K. & Croizier, L. (1989). A physical map of *Spodoptera littoralis* B-type nuclear polyhedrosis virus genome. *Arch Virol* **104**, 145–151.

Croizier, G., Croizier, L., Argaud, O. & Poudevigne, D. (1994). Extension of *Autographa californica* nuclear polyhedrosis virus host range by interspecific replacement of a short DNA sequence in the p143 helicase gene. *Proc Natl Acad Sci U S A* **91**, 48–52.

Escribano, A., Williams, T., Goulson, D., Cave, R. D., Chapman, J. W. & Caballero, P. (1999). Selection of a nucleopolyhedrovirus for control of *Spodoptera frugiperda* (Lepidoptera: Noctuidae): structural, genetic, and biological comparison of four isolates from the Americas. *J Econ Entomol* **92**, 1079–1085.

Faktor, O., Toister-Achituv, M. & Kamensky, B. (1995). Identification and nucleotide sequence of an ecdysteroid UDP-glucosyltransferase gene of *Spodoptera littoralis* multicapsid nuclear polyhedrosis virus. *Virus Genes* **11**, 47–52.

Gelernter, W. D. & Federici, B. A. (1986). Isolation, identification and determination of virulence of a nuclear polyhedrosis virus from the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environ Entomol* **15**, 240–245.

Greene, G. L., Leppla, N. C. & Dickerson, W. A. (1976). Velvetbean caterpillar: a rearing procedure and artificial medium. *J Econ Entomol* **69**, 487–488.

Gröner, A. (1986). Specificity and safety of baculoviruses. In *The Biology of Baculoviruses: Biological Properties and Molecular Biology*, pp. 177–202. Edited by R. R. Granados & B. A. Federici. San Diego: Academic Press.

Heldens, J. G., Broer, R., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1997). Identification and functional analysis of a non-*hr* origin of DNA replication in the genome of *Spodoptera exigua* multicapsid nucleopolyhedrovirus. *J Gen Virol* **78**, 1497–1506.

Hughes, P. R. & Wood, H. A. (1981). A synchronous peroral technique for the bioassay of insect viruses. *J Invertebr Pathol* **37**, 154–159.

Ijkel, W. F., van Strien, E. A., Heldens, J. G., Broer, R., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1999). Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. *J Gen Virol* **80**, 3289–3304.

Kamita, S. G., Maeda, S. & Hammock, B. D. (2003). High-frequency homologous recombination between baculoviruses involves DNA replication. *J Virol* **77**, 13053–13061.

Kondo, A. & Maeda, S. (1991). Host range expansion by recombination of the baculoviruses *Bombyx mori* nuclear polyhedrosis virus and *Autographa californica* nuclear polyhedrosis virus. *J Virol* **65**, 3625–3632.

Kool, M., Ahrens, C. H., Goldbach, R. W., Rohrmann, G. F. & Vlak, J. M. (1994). Identification of genes involved in DNA replication of the *Autographa californica* baculovirus. *Proc Natl Acad Sci U S A* **91**, 11212–11216.

LaCount, D. J. & Friesen, P. D. (1997). Role of early and late replication events in induction of apoptosis by baculoviruses. *J Virol* **71**, 1530–1537.

López-Ferber, M., Simon, O., Williams, T. & Caballero, P. (2003). Defective or effective? Mutualistic interactions between virus genotypes. *Proc R Soc Lond B Biol Sci* **270**, 2249–2255.

Lu, A. & Miller, L. K. (1997). Regulation of baculoviruses late and very late gene expression. In *The Baculoviruses*, pp. 193–216. Edited by L. K. Miller. New York: Plenum.

Maeda, S., Kamita, S. G. & Kondo, A. (1993). Host range expansion of *Autographa californica* nuclear polyhedrosis virus (NPV) following recombination of a 0·6-kilobase-pair DNA fragment originating from *Bombyx mori* NPV. *J Virol* **67**, 6234–6238.

Martin, O. & Croizier, G. (1997). Infection of a *Spodoptera frugiperda* cell line with *Bombyx mori* nucleopolyhedrovirus. *Virus Res* **47**, 179–185.

Miller, L. K. (1997). Baculovirus interaction with host apoptotic pathways. *J Cell Physiol* **173**, 178–182.

Miller, L. K. & Lu, A. (1997). The molecular basis of baculovirus host range. In *The Baculoviruses*, pp. 217–235. Edited by L. K. Miller. New York: Plenum.

Monsma, S. A., Oomens, A. G. & Blissard, G. W. (1996). The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. *J Virol* **70**, 4607–4616.

Moscardi, F. (1999). Assessment of the application of baculoviruses for control of Lepidoptera. *Annu Rev Entomol* **44**, 257–289.

Muñoz, D., Castillejo, J. I. & Caballero, P. (1998). Naturally occurring deletion mutants are parasitic genotypes in a wild-type nucleopolyhedrovirus population of *Spodoptera exigua*. *Appl Environ Microbiol* **64**, 4372–4377.

Murillo, R., Muñoz, D. & Caballero, P. (2003). Host range and biological activity of three *Spodoptera* nucleopolyhedrovirus genotypic variants and the effect of Tinopal LPW on the most active variant. *Int J Pest Manag* **49**, 147–153.

Prikhod'ko, E. A. & Miller, L. K. (1996). Induction of apoptosis by baculovirus transactivator IE1. *J Virol* **70**, 7116–7124.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Simón, O., Williams, T., López-Ferber, M. & Caballero, P. (2004). Structure of a *Spodoptera frugiperda* nucleopolyehdrovirus population: high prevalence of *per os* non-infective deletion mutants. *Appl Environ Microbiol* **70** (in press).

Thiem, S. M. (1997). Prospects for altering host range for baculovirus bioinsecticides. *Curr Opin Biotechnol* **8**, 317–322.

Thiem, S. M., Du, X., Quentin, M. E. & Berner, M. M. (1996). Identification of baculovirus gene that promotes *Autographa californica* nuclear polyhedrosis virus replication in a nonpermissive insect cell line. *J Virol* **70**, 2221–2229.

Tumilasci, V. F., Leal, E., Zanotto, P. M., Luque, T. & Wolff, J. L. (2003). Sequence analysis of a 5·1 kbp region of the *Spodoptera frugiperda* multicapsid nucleopolyhedrovirus genome that comprises a functional ecdysteroid UDP-glucosyltransferase (*egt*) gene. *Virus Genes* **27**, 137–144.

Washburn, J. O., Kirkpatrick, B. A. & Volkman, L. E. (1996). Insect protection against viruses. *Nature* **383**, 767.

Washburn, J. O., Haas-Stapleton, E. J., Tan, F. F., Beckage, N. E. & Volkman, L. E. (2000). Co-infection of *Manduca sexta* larvae with

polydnavirus from *Cotesia congregata* increases susceptibility to fatal infection by *Autographa californica* M Nucleopolyhedrovirus. *J Insect Physiol* **46**, 179–190.

Yanase, T., Yasunaga, C., Hara, T. & Kawarabata, T. (1998a). Coinfection of *Spodoptera exigua* and *Spodoptera frugiperda* cell lines with the nuclear polyhedrosis viruses of *Autographa californica* and *Spodoptera exigua*. *Intervirology* **41**, 244–252.

Yanase, T., Yasunaga, C. & Kawarabata, T. (1998b). Replication of *Spodoptera exigua* nucleopolyhedrovirus in permissive and non-permissive lepidopteran cell lines. *Acta Virol* **42**, 293–298.

Zhang, P., Yang, K., Dai, X., Pang, Y. & Su, D. (2002). Infection of wild-type *Autographa californica* multicapsid nucleopolyhedrovirus induces *in vivo* apoptosis of *Spodoptera litura* larvae. *J Gen Virol* **83**, 3003–3011.