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* (SMNPV), *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 SMNPV, 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 SMNPV 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 SMNPV 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 SMNPV 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 SMNPV, hereafter named SfNIC (Escríbano *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 SMNPV (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 10^6–10^9 OBs ml⁻¹. Each larva was injected with 8 μl occlusion derived virions (ODVs) obtained after alkaline treatment of the corresponding concentration of OBs (1:1:5 OBs:0.5 M Na₂CO₃:H₂O by volume) (López-Carabias *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 Na₂CO₃ 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 *Pvu*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
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. Second, SeUS1 infection in the three Spodoptera host species was followed in detail. The transcriptions signals of ie-0, DNA polymerase (an early gene), chitinase

Table 1. Oligonucleotides used in the study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (position in the genome)</th>
<th>Amplification purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sfie0.1</td>
<td>5’-TACGCTCGAGATGAGTATTAATCATGATTTC-3’</td>
<td>Forward and reverse primers that amplified 500 bp central fragment of early transcribed SfNIC ie-0 gene</td>
</tr>
<tr>
<td>Sfie0.2</td>
<td>5’-CTACTCGAGTCTGCAATGTTACACT-3’</td>
<td>Reverse and forward primers that amplified 510 bp fragment in 3’ extremity of early transcribed SeUS1 ie-0 gene</td>
</tr>
<tr>
<td>Seie0.1</td>
<td>5’-CTATAACTCGAGCTGGTGTT-3’</td>
<td>Forward and reverse primers that amplified 319 bp fragment in 5’ extremity of early transcribed SfNIC DNA polymerase gene</td>
</tr>
<tr>
<td>Seie0.2</td>
<td>5’-ATCGTCTCTGATACCCGGAG-3’</td>
<td>Forward and reverse primers that amplified 339 bp fragment in 5’ extremity of late transcribed SfNIC chitinase gene</td>
</tr>
<tr>
<td>SeDnPol.1</td>
<td>5’-ATGACTTCTCTGTCGTCGTC-3’</td>
<td>Forward and reverse primers that amplified 319 bp fragment in 5’ extremity of very late transcribed SfNIC polyhedrin gene</td>
</tr>
<tr>
<td>SeDnPol.2</td>
<td>5’-TACGACGCTGTTAGGCTGTT-3’</td>
<td>Reverse and forward primers that amplified 370 bp fragment in 3’ extremity of early transcribed SpliM2 egt</td>
</tr>
</tbody>
</table>
Detection of SINIC, 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 SINIC, SeUS1 and/or SpliM2 DNA in larvae inoculated with SINIC, SeUS1 or SpliM2, or with a virus mixture (SINIC/SeUS1, SeUS1/SpliM2 or SINIC/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 SINIC 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⁻². 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 SINIC, 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.

SINIC 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 (SINIC, SeUS1 and SpliM2) initiated an infection in all three host species tested (S. frugiperda, S. exigua and S. littoralis). Following oral inoculation with OBs, SINIC-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.

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**Fig. 1.** Detection of early transcripts of SINIC, SeUS1 and SpliM2 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 SINIC 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 SINIC 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...
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* inoculation.
inoculation (Fig. 4a), but 4-7 times greater by intrahaemo-
colic 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 (~1.3 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 abun-
dance 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 intrahaemoocolic injection
(Fig. 4b). In general, DNA replication of SeUS1 in hetero-
logous 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 hetero-
logous 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 combi-
nations of SeUS1, SfNIC and SpliM2 was analysed with PstI
data not shown). Viral DNAs extracted from larvae inoculated
can 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 intrahaemoocolic injection
(data not shown).

In the second inoculation, S. exigua, S. frugiperda or S. littor-
alis larvae were inoculated with OBs of SfNIC/SeUS1 or
SpliM2/SeUS1 at a ratio of 1 : 100,000. A REN analysis with
PstI 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 quan-
tity 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
geno-
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 a 1:1 mixture or in a ratio of 1:100,000

<table>
<thead>
<tr>
<th>Event</th>
<th>Propagating host</th>
<th>Se</th>
<th>Sf</th>
<th>Spli</th>
<th>Se + Sf</th>
<th>Se + Spli</th>
<th>Sf + Spli</th>
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<td>Early gene expression</td>
<td>S. exigua</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td></td>
<td>S. frugiperda</td>
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<td>+++</td>
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<td>ND</td>
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<td>ND</td>
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<tr>
<td></td>
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<td>Viral DNA replication</td>
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<td></td>
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<td>S. frugiperda</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S. littoralis</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Detected by PCR</td>
<td>S. exigua</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>S. frugiperda</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>S. littoralis</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Very late gene expression</td>
<td>S. exigua</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>S. frugiperda</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>S. littoralis</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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.

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...
ultimately cleared. The time-course of SeUS1 infection in cyte aggregations transformed infected cells into melanized 72 h p.i., haemocytes surround infected cells and haemocyte the number of infection foci of AcMNPV was detected. By expression. However, in heterologous hosts, a decline in infection of AcMNPV began at the same time as in permissive hosts, and secondary infections in midgut-associated tracheae species, primary infection of midgut columnar cells by AcMNPV was incapable of replication in these cells (Maeda et al., 1993; Crozier 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 & Friessen, 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 AcNPV 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 haemo- cyte 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.

ACKNOWLEDGEMENTS

We thank L. Crozier, J. Jolivet, N. Grard and S. Gutierrez (INRA) and L. Maquirriain (UPNA) for technical assistance, and B. Limier (INRA) and N. Gorria (UPNA) for insect rearing. The study received financial support from CICYT projects AGL2000-0840-C03-03, AGL2002-04320-C02-01 and a studentship awarded to O.S.

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