Spodoptera exigua multicapsid nucleopolyhedrovirus deletion mutants generated in cell culture lack virulence in vivo

Jacobus G. M. Heldens, 1 Elisabeth A. van Strien, 1 Alphons M. Feldmann, 2 Peter Kulcsár, 1 Delia Munoz, 3 Douglas J. Leisy, 4 Douwe Zuidema, 1 Rob W. Goldbach 1 and Just M. Vlak 1

1 Department of Virology, Wageningen Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands
2 Institute for Plant Protection Research, Binnenhaven 5, 6709 PD Wageningen, The Netherlands
3 Departamento Produccion Agraria, Universidad Pública de Navarra, Campus de Arrosadia, 310006 Pamplona, Navarra, Spain
4 Department of Agricultural Chemistry, Oregon State University, Corvallis, OR 97331, USA

The baculovirus Spodoptera exigua multicapsid nucleopolyhedrovirus (SeMNPV) has high potential for development as a bio-insecticide for control of the beet armyworm (S. exigua). It is highly infectious for S. exigua larvae and its host range is very narrow. A prerequisite for such application is the possibility of growing this virus in large quantities, e.g. in insect cell lines. It was observed, however, that polyhedra of SeMNPV plaque-purified in Se-UCR1 cells did not cause larval mortality or morbidity when fed to S. exigua larvae. As this suggested a genetic alteration in in vitro produced SeMNPV, comparative restriction analysis of in vitro and in vivo produced SeMNPV DNA was performed. The restriction patterns of viral DNA from several different plaques always differed from that of the wild-type in the same way, suggesting that a large, single deletion had occurred in the in vitro produced viral genome. In order to localize this deletion more precisely a detailed physical map of the wild-type SeMNPV genome was constructed, using the restriction endonucleases XbaI, BamHI, BglII, PstI, SstI, HindIII and Spel. In addition, the entire SeMNPV genome was cloned into a library containing five overlapping cosmids and a plasmid library. About 80 restriction sites were located and the orientation of the map was set according to the location of the polyhedrin and p10 genes. The approximate size of the viral genome was 134 kbp. Based on this map it could be established that mutant SeMNPV, obtained by passage in cell culture, contained a single deletion of approximately 25 kbp between map units 12.9 and 32.3.

Introduction

The beet army worm (Spodoptera exigua; Lepidoptera, Noctuidae) is an agriculturally important pest insect in (sub)tropical regions of the Northern hemisphere and in greenhouses. The insect is resistant to many commonly used chemical insecticides. Recently a baculovirus of this insect, S. exigua multicapsid nucleopolyhedrovirus (SeMNPV) has been registered in several countries as a biological insecticide (Smits & Vlak, 1994). SeMNPV is an attractive bio-insecticide since the virus has a narrow host-range and is relatively virulent compared to other baculoviruses (Smits et al., 1988). It may take several days after infection until the larvae stop feeding. The insecticidal properties of SeMNPV, in particular the speed of action, might be improved by genetic engineering. Successful attempts have been reported for Autographa california MNPV (AcMNPV) via the introduction of insect-specific neurotoxin genes into the genome or the construction of ecdysteroid-UDP-glucosyltransferase (egt) gene deletion mutants (Stewart et al., 1991; McCutchen et al., 1991; Tomalski & Miller, 1991; O’Reilly & Miller, 1991). A prerequisite for successful genetic modification of SeMNPV is the availability of a physical and genetic map. Furthermore, a cell line able to support virus replication would facilitate the engineering of SeMNPV and the structural and functional analysis of its genome.

Author for correspondence: Just M. Vlak.
Fax +31 317 484820. e-mail just.vlak@medew.viro.wau.nl

Present address: Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Karolina ut 29, H-1113 Budapest, Hungary
SeMNPV has a circular double-stranded DNA genome of about 130 kbp (Caballero et al., 1992). Restriction fragment length polymorphism in several SeMNPV isolates has been reported (Caballero et al., 1992), but a detailed physical map of the viral genome is not yet available. Three SeMNPV genes have been characterized and provisionally localized on genome fragments: polyhedrin (van Strien et al., 1992), p10 (Zuidema et al., 1993) and ubiquitin (van Strien et al., 1996). Two insect cell lines derived from *S. exigua* have been described that support SeMNPV replication (Gelernter & Federici, 1986; Hara et al., 1993, 1994). Preliminary experiments on the replication of SeMNPV in the cell line Se-UCR1 indicated that the budded virus (BV) was highly infectious for these cells, but that the polyhedra produced lacked infectivity for insects. This phenomenon could be correlated with a deletion of about 25 kbp from the SeMNPV genome. In this report, a physical map of the SeMNPV genome for seven restriction endonucleases is described allowing the genetic analysis of *in vivo* and *in vitro* produced SeMNPV and the localization of the deletion in the latter.

**Methods**

**Virus, insects and cells.** *Spodoptera exigua* MNPV (SeMNPV/US) (Gelernter & Federici, 1986a) was obtained from B.A. Federici, Department of Entomology, University of California at Riverside, Riverside, Calif., USA, in the form of polyhedra. The virus was propagated in fourth instar larvae of *S. exigua* (Smits et al., 1988). Larvae were infected by contamination of artificial diet with polyhedra. Haemolymph from SeMNPV-infected insects was used as a source of BV for the infection of cultured *Spodoptera exigua* cells (Gelernter & Federici, 1986b). This cell line (Se-UCR1), obtained from B.A. Federici, was maintained in plastic cell culture flasks in TNM-FH medium (Hink, 1970) supplemented with 10% fetal calf serum. *S. exigua* fourth instar larvae were infected with a dose of 10⁴ SeMNPV polyhedra/ml sufficient to kill 99% of the larvae. Four days post-infection (p.i.) haemolymph was obtained from a cut proleg and used to infect 10⁶ Se-UCR1 cells. Plaque purification of SeMNPV BV was carried out according to the procedures described by Summers & Smith (1987).

**DNA isolation, Southern blot hybridization and molecular cloning.** Wild-type SeMNPV DNA was obtained from alkali-liberated virions purified after alkali treatment of polyhedra (PD) followed by sucrose gradient centrifugation (SeMNPV PD-DNA) (Caballero et al., 1992). Alternatively, viral DNA was isolated from SeMNPV BV (SeMNPV BV-DNA) according to the procedures described by Summers & Smith (1987). SeMNPV (BV- or PD-derived) or cosmid DNA thereof was digested with restriction endonucleases and electrophoresed in 0.8% agarose gels. transfected to Hybond N+ nylon membranes (Amersham) and hybridized with ³²P-labelled DNA fragments of SeMNPV according to procedures described by Sambrook et al. (1989). Xbal-digested SeMNPV fragments (C through R) were isolated from agarose gels by the freeze-squeeze method (Sambrook et al., 1989) and cloned into pUC vectors.

SeMNPV PD-DNA was partially digested with the restriction enzyme *Sac*III to generate fragments of about 35 kbp. These fragments were ligated into BamHI digested and dephosphorylated pWE15 (Stratagene). Cosmid ligation mixes were packaged *in vitro* into λ phage heads and transduced into *Escherichia coli* DH5α cells according to the protocols of the manufacturer (Stratagene). Ampicillin resistant colonies were selected and cosmid DNA was isolated by a rapid minipreparation procedure (Sambrook et al., 1989). Maxipreparation of cosmid DNA was carried out according to the Qiaquent 100 protocol.

**Dot blot analysis.** Cosmid and plasmid DNA was isolated according to the minipreparation method described and 1/10th of the yield of DNA was denatured in 200 μl 200 mM-NaOH for 10 min. The dot blot apparatus (Bio-Rad) was assembled and the wells were washed with 400 μl 2 M-NaCl before the denatured cosmid DNA was applied to the filter (Hybond N+/ Amersham). The membrane was removed and air-dried prior to baking for 2 h at 80 °C. Hybridization was performed with ³²P-labelled DNA fragments according to Sambrook et al. (1989).

**Results**

**Infectivity of *in vivo* and *in vitro* produced polyhedra**

Fourth instar *S. exigua* larvae were orally infected with polyhedra produced in insects (*in vivo*) or derived from plaque-purified, *in vitro* produced SeMNPV. Larvae infected with *in vivo* produced SeMNPV polyhedra died and decayed normally. However, larvae that were infected with SeMNPV polyhedra derived from *in vitro* produced, plaque-purified SeMNPV pupated normally and did not die. BV from plaque-purified SeMNPV was highly infectious for Se-UCR1 cells. When injected into the haemolymph this BV did not cause morbidity or mortality. Haemolymph isolated from larvae infected with *in vitro* produced, plaque-purified SeMNPV polyhedra was highly infectious for Se-UCR1 cells indicating that the mutant virus does pass the larval midgut cells and that it undergoes at least one round of replication. These results suggest that the *in vitro* produced SeMNPV is genetically altered resulting in the loss of *in vivo* mortality and morbidity. Therefore, the DNA of SeMNPV produced *in vitro* and *in vivo* was subjected to restriction enzyme analysis to further study this phenomenon.

**Comparison of *in vivo* and *in vitro* produced SeMNPV DNA**

The DNA of *in vivo* and *in vitro* produced SeMNPV was analysed with the restriction enzymes Xbal and *PstI* (Fig. 1) and others (not shown). *In vitro* produced SeMNPV fragment Xbal-A was absent from *in vivo* produced SeMNPV DNA and a new smaller fragment between Xbal-B and -C was present (Fig. 1, lanes 3 and 4). Likewise, fragments *PstI*-C and -D were no longer present in the *in vitro* produced SeMNPV DNA (Fig. 1, lanes 1 and 2). Various independently isolated plaques from repeated *in vitro* infections showed identical restriction profiles. When BVs were analysed from Se-UCR1 cells infected with haemolymph-derived BVs (first *in vitro* passage) and compared to *in vitro* produced SeMNPV, several submolar fragments were detected in all the restriction profiles (data not shown). When these BVs were further passaged over Se-UCR1 cells twice more and plaque-purified, submolar bands were no longer observed even by Southern blot hybridization (Fig. 1). These results suggest that a large deletion had occurred in the
passaging of haemolymph-derived BV in cell culture and that genes present in the deletion are essential for virus virulence in vivo but not in vitro.

**Physical mapping of the SeMNPV genome**

To locate the deletion in the SeMNPV genome, a physical map was constructed. Polyhedra derived SeMNPV DNA was digested with the restriction enzymes XbaI, BamHI, BglII, PstI, SstI, HindIII and SpeI, and separated by agarose gel electrophoresis (Fig. 2). Fragment sizes were estimated by comparison with size markers in adjacent lanes and ranged from approximately 36 kbp for the XbaI-A fragment to 1-1 kbp for the SstI-H fragment (Table 1). Smaller fragments for these enzymes were not detected with this technique or by Southern blot hybridization (data not shown). By totalling the sizes of the fragments generated by the respective restriction enzymes the average size of the entire SeMNPV genome was calculated to be 134±1 kbp. This is about the size of the genome of AcMNPV (Ayres et al., 1994).

SeMNPV PD-DNA was digested to completion with XbaI and fragments C through R (11.5 through 1.7 kbp) were cloned into plasmid pUC19. The fragments XbaI-A and XbaI-B (35.6 and 17.5 kbp, respectively) were too large to be inserted into pUC plasmid vectors. In order to clone the entire viral genome, a cosmid library was constructed. A set of five cosmid clones from the library spanning the entire viral genome except a small region located in the XbaI-D fragment (Fig. 3b) was selected from the library by dot blotting cosmids and Southern hybridization with 32P-labelled XbaI fragments (A through R) of SeMNPV-DNA. Cross-blot hybridization provided data about overlapping cosmid inserts. The occurrence of contiguous overlapping fragments in these cosmids confirmed the circularity of the SeMNPV genome.

Physical maps of the five cosmid clones were constructed by single- and double-digestions using the seven restriction enzymes mentioned above and Southern hybridization. By compilation of the physical maps of the five cosmid clones a complete physical map of the viral genome containing about 80 restriction endonuclease sites was derived (Fig. 3). This map was further validated by hybridizing individual 32P-labelled XbaI fragments to Southern blots of SeMNPV PD-DNA digested (single and double) with the seven restriction enzymes. The adenine residue at the translational initiation codon of the polyhedrin gene, upstream of the junction between the XbaI-D and -R fragments (van Strien et al., 1992) was designated as the zero point of the physical map (Fig. 3a, c). The orientation of the map was set by the location of the p10 gene of SeMNPV (Zuidema et al., 1993) on the XbaI-H fragment at the right side of the map and is in agreement with the convention for linearized baculovirus maps (Vlak & Smith, 1982).
Fig. 2. Electrophoresis of SeMNPV PD DNA produced in vivo and digested with Xbol (lane 2), PstI (lane 3), BglII (lane 4), BamHI (lane 5), SpeI (lane 6), SstI (lane 7) and HindIII (lane 8) in a 0.8% agarose gel. HindIII-digested λ DNA (lane 1) served as a size standard. Restriction fragments are lettered in order of size.
Table 1. Fragment sizes of occlusion body derived SeMNPV DNA

Fragments were generated by the restriction enzymes BamHI, BglII, PstI, XbaI, SpeI, HindIII and SstI; fragment sizes are indicated in kbp. Restriction fragments that are altered in mutant SeMNPV DNA are either deleted (d) or truncated (*). Truncated fragments and their sizes need to be summed to obtain the actual fragment size in the deletion mutant. The truncated fragments are displayed in the row of the fragment from which they originate.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>XbaI</th>
<th>PstI</th>
<th>BamHI</th>
<th>HindIII</th>
<th>SpeI</th>
<th>BglII</th>
<th>SstI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>35-6 (13-6)</td>
<td>22-0</td>
<td>29-6 (25-2*)</td>
<td>30-7</td>
<td>27-6 (18-0*)</td>
<td>36-1 (11-7)</td>
<td>29-2</td>
</tr>
<tr>
<td>B</td>
<td>17-5</td>
<td>21-0</td>
<td>24-0 (23-0*)</td>
<td>29-2 (17-0*)</td>
<td>22-4</td>
<td>21-0</td>
<td>26-3</td>
</tr>
<tr>
<td>C</td>
<td>11-5</td>
<td>13-8 (d)</td>
<td>25-8</td>
<td>27-3 (14-8*)</td>
<td>39-5</td>
<td>19-5</td>
<td>21-8</td>
</tr>
<tr>
<td>D</td>
<td>10-7</td>
<td>11-5 (d)</td>
<td>16-4</td>
<td>26-0</td>
<td>16-5</td>
<td>15-3</td>
<td>20-0</td>
</tr>
<tr>
<td>E</td>
<td>8-0</td>
<td>9-0</td>
<td>14-2</td>
<td>13-0</td>
<td>15-8 (6-2*)</td>
<td>10-5</td>
<td>18-6 (4-5*)</td>
</tr>
<tr>
<td>F</td>
<td>6-2</td>
<td>8-7</td>
<td>14-0 (d)</td>
<td>8-5</td>
<td>12-5</td>
<td>6-4</td>
<td>12-5 (2-2*)</td>
</tr>
<tr>
<td>G</td>
<td>5-8</td>
<td>8-7</td>
<td>4-5</td>
<td>9-3</td>
<td>6-2</td>
<td>4-5</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>5-2</td>
<td>7-6</td>
<td>1-2 (d)</td>
<td>5-3 (d)</td>
<td>5-3</td>
<td>1-1</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5-0</td>
<td>6-8</td>
<td>4-4</td>
<td>5-0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>4-6</td>
<td>4-8</td>
<td>4-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>4-0</td>
<td>4-6</td>
<td>3-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>3-9</td>
<td>3-9</td>
<td>2-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>3-5</td>
<td>3-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>3-3</td>
<td>3-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>3-0</td>
<td>2-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>2-1</td>
<td>1-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q</td>
<td>2-0</td>
<td>1-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>1-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ</td>
<td>133-6</td>
<td>134-4</td>
<td>133-7</td>
<td>134-7</td>
<td>133-6</td>
<td>135-4</td>
<td>134-0</td>
</tr>
</tbody>
</table>

Mapping of the deletion in DNA of SeMNPV produced in vitro

When the sizes of the DNA fragments of SeMNPV produced in vitro were summed, the genome size was calculated to be about 110 kbp. This is approximately 25 kbp shorter than the DNA of the in vivo produced SeMNPV. The fact that only XbaI-A (35-6 kbp) disappeared and a new fragment of about 14 kbp was identified suggests that the deletion is contiguous. Based on the physical map of wild-type SeMNPV-DNA the deletion in the DNA of the in vitro produced SeMNPV could be approximately located. This was done by hybridization of the latter DNA with plasmids overlapping the deletion. Fragments PstI-C and -D, BamHI-F and -H and SpeI-H were absent, whereas fragments XbaI-A, BamHI-A and -B, BglII-A, SpeI-A and -E, SstI-E and -F and HindIII-A and -D hybridized to new (junction) fragments (Table 1). Since the size of these junction fragments could be determined and compared, the deletion could be approximately located between map units 12-9 and 32-3 (Fig. 3).

Discussion

Upon serial passaging of AcMNPV in Spodoptera frugiperda cells at high multiplicity defective interfering viruses arise with deletions up to 50 kbp (Kool et al., 1991). However, a deletion of about 25 kbp of the viral genome within the first passage, as is the case for SeMNPV, has not been observed previously. Moreover, it is not reported that these early arising defective interfering particles of AcMNPV are defective in larval infection. Studies are currently underway using in vitro produced SeMNPV polyhedra of passage one, five and ten to determine virulence, LD50 and LT50 in S. exigua larvae (E. M. Colbers & J. M. Vlak, unpublished).

The question is whether the SeMNPV deletion mutant is defective and needs the co-infection of a wild-type helper virus at a very low concentration to complement the functions of the deleted genes, or whether it is indeed a viable virus, which could be obtained by plaque purification. If a helper virus is involved, its concentration is below the level of detection by Southern hybridization. The presence of a helper virus is unlikely since in vitro produced SeMNPV polyhedra do not cause any effect in vivo nor does the injection of BV into the haemolymph. The assumption that no helper virus is involved implies that no genes involved in virus encapsidation or replication are located on the deleted fragment. Plaque purification of wild-type virus is therefore impossible since upon multiple passaging in the Se-UCR1 cell culture the deletion of the viral genome apparently starts immediately and proceeds continuously until the final stable deletion mutant is generated. Since plaque-purified SeMNPV polyhedra do not cause disease in the insect, it could be concluded that the 25 kbp deletion contains information that is important for virus virulence in vivo. However, it cannot be excluded that mutations elsewhere in the genome that do not alter the...
Fig. 3. Physical map of SeMNPV PD-DNA. (a) Physical map of the SeMNPV-PD genome for the restriction endonucleases BamHI, BglII, PstI, XbaI, SpeI, HindIII and SstI with the position of the site in map units. (b) Location of the set of five cosmids (7, 17, 22, 24 and 32) on the SeMNPV genome. (c) Setting of the map units and kbp scale. The zero point is at the A of the ATG of the polyhedrin gene which located upstream of the junction between the XbaI-D and -R fragments. (d) The bidirectional arrow spans the deletion in the genome of in vitro produced SeMNPV (between map units 12.9 and 32.3).
restriction enzyme profiles are contributing to or causing the loss of in vivo activity of mutant SeMNPV. In vivo rescue of the deletion mutant virus using cosmids 24 and 17 (Fig. 3) should answer this question as this would restore virulence for insects.

Limited nucleotide sequence analysis of the segment deleted in the defective viral genome revealed that it encodes either genes which are involved in larval decay, such as cathepsin (Ohkawa et al., 1994) and chitinase (Hawtin et al., 1995), or insect hormone regulation, such as egt (D. Zuidema and others, unpublished). In cell culture, no apparent selection pressure exists on the presence of either type of the above described genes in the viral genome. Therefore, these genes may be deleted spontaneously out of the SeMNPV genome when the virus is maintained in cell culture. Lack of infectivity of in vitro produced SeMNPV is a major limitation towards the application of cell culture for large scale operations.

Hara et al. (1993) reported that SeMNPV produced in the Se-301 cell line was still infectious to larvae. Rearrangements present in plaque-purified SeMNPV-DNA as compared to wild-type were not noted. This may suggest that a 'cell factor' may be involved in the generation of the above described genes in the viral genome. Therefore, these genes may be deleted spontaneously out of the SeMNPV genome when the virus is maintained in cell culture. Lack of infectivity of in vitro produced SeMNPV is a major limitation towards the application of cell culture for large scale operations.

When the genetic organization of SeMNPV is compared with that of Orgyia pseudotsugata MNPV (OpMNPV) and of AcMNPV with respect to the location of the polyhedrin and p10 genes, the SeMNPV polyhedrin gene (van Strien et al., 1992) is transcribed in the same direction as the AcMNPV polyhedrin gene (Ayres et al., 1994), but in the opposite direction to the OpMNPV polyhedrin gene (Leisy et al., 1992a, b). The distances between these two genes in the various viruses are also different, being 19.3 kbp for AcMNPV (Ayres et al., 1994), 22.3 kbp for OpMNPV (Leisy et al., 1992a, b) and 11.6 kbp for SeMNPV, respectively (van Strien et al., 1992; Zuidema et al., 1993). This suggests that between these baculoviruses the genetic organization is not entirely conserved despite the similar length of their genomes (132 kbp).

The genome size of the SeMNPV-US isolate (134 kbp) described here is in agreement with previously reported estimated genome sizes of SeMNPV-US and several SeMNPV Spanish field isolates (132 kbp) (Caballero et al., 1992), but not with the size Hara et al. (1995) reported recently for Japanese plaque-purified and field isolates of SeMNPV (122 and 105 kbp, respectively).

Southern blot hybridization with homologous fragments did not show any cross-hybridization with any fragment in the SeMNPV genome. However, it cannot be ruled out that small and/or imperfect homologous regions are interspersed throughout the SeMNPV genome, and could not be detected with the methods that were used in this study. Recently, small hr-like sequences were detected by sequence analysis of SeMNPV (R. Broer and others, unpublished).

The availability of a cosmid library and a physical map of SeMNPV-DNA constructed for several restriction enzymes now allows a detailed study of the SeMNPV genome, including its replication mechanism and the characterization of genes located on the deleted segment. Furthermore, the physical map of SeMNPV-DNA facilitates the study of the genetic relatedness of this virus with other baculoviruses.

We thank Dr P.J. Krell for critical reading of the manuscript and Ms M. Usmany for excellent technical assistance in cell culture experiments. This research was supported by the Dutch-Israeli Agricultural Research Program grant no. 93/20. P.K. was supported by a senior fellowship from the Wageningen Agricultural University. I.G.M.H. and E.A.V.S. are graduate students of the Research School Production Ecology of the Agricultural University Wageningen.

References


Received 18 June 1996; Accepted 13 August