Journal of General Virology (2000), **81**, 2545–2554. Printed in Great Britain

Isolation of a *Spodoptera exigua* baculovirus recombinant with a 10·6 kbp genome deletion that retains biological activity

Xiaojiang Dai,[1,2] József P. Hajós,[1,3] Nina N. Joosten,[1] Monique M. van Oers,[1] Wilfred F. J. IJkel,[1] Douwe Zuidema,[1] Yi Pang[2] and Just M. Vlak[1]

[1] Laboratory of Virology, Wageningen University and Research Centre, Binnenhaven 11, 6709 PD Wageningen, The Netherlands
[2] State Key Laboratory for Biocontrol and Institute of Entomology, Zhongshan University, Guangzhou 510275, People's Republic of China
[3] Institute of Enzymology, Biological Research Centre, Hungarian Academy of Sciences, PO Box 7, 1518 Budapest, Hungary

When *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) is grown in insect cell culture, defective viruses are generated. These viruses lack about 25 kbp of sequence information and are no longer infectious for insects. This makes the engineering of SeMNPV for improved insecticidal activity or as expression vectors difficult to achieve. Recombinants of *Autographa californica* MNPV have been generated in insects after lipofection with viral DNA and a transfer vector into the haemocoel. In the present study a novel procedure to isolate SeMNPV recombinants was adopted by alternate cloning between insect larvae and cultured cells. The *S. exigua* cell line Se301 was used to select the putative recombinants by following a green fluorescent protein marker inserted in the *p10* locus of SeMNPV. Polyhedra from individual plaques were fed to larvae to select for biological activity. In this way an SeMNPV recombinant (SeXD1) was obtained with the speed of kill improved by about 25%. This recombinant lacked 10593 bp of sequence information, located between 13·7 and 21·6 map units of SeMNPV and including *ecdysteroid UDP glucosyl transferase*, *gp37*, *chitinase* and *cathepsin* genes, as well as several genes unique to SeMNPV. The result indicated, however, that these genes are dispensable for virus replication both *in vitro* and *in vivo*. A mutant with a similar deletion was identified by PCR in the parental wild-type SeMNPV isolate, suggesting that genotypes with differential biological activities exist in field isolates of baculoviruses. The generation of recombinants *in vivo*, combined with the alternate cloning between insects and insect cells, is likely to be applicable to many baculovirus species in order to obtain biologically active recombinants.

Introduction

The beet army worm *Spodoptera exigua* causes extensive economic losses in many cultivated crops throughout the temperate and subtropical regions of the Northern hemisphere and in greenhouses. The insect is resistant to many commonly used chemical insecticides. *S. exigua* multicapsid nucleopolyhedrovirus (SeMNPV) is an attractive bio-insecticide since the virus is monospecific to the beet army worm and highly virulent as compared to other baculoviruses (Smits *et al.*, 1988). It has also been commercialized as a bio-insecticide (Smits & Vlak, 1994). However, further improvements in the biological activity of SeMNPV are sought, either by strain selection (Muñoz *et al.*, 1998) or by genetic engineering.

The molecular genetics of SeMNPV have been relatively well studied. A detailed physical map has been constructed (Heldens *et al.*, 1996) and a number of SeMNPV genes have been characterized in detail (van Strien *et al.*, 1992, 1996, 1997; Zuidema *et al.*, 1993; Heldens *et al.*, 1997). Recently the complete sequence and gene organization of the SeMNPV genome have been reported (IJkel *et al.*, 1999). However, the molecular basis for specificity and virulence has not yet been revealed.

Several cell lines have been derived from *S. exigua*, such as SeUCR (Gelernter & Federici, 1986*a*), Se301 (Hara *et al.*, 1995*b*) and IZD2109 (B. Möckel, personal communication), and susceptibility to SeMNPV has been reported (Hara *et al.*,

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

0001-7048 © 2000 SGM

1993, 1995*a*). However, when SeMNPV is grown in insect cell culture defective viruses are quickly generated (Heldens *et al.*, 1996). The majority of these viruses lack about 25 kbp of sequence information and are no longer infectious for insects. The deletion is located approximately between 12·9 and 32·3 map units (m.u.) and encompasses the SeMNPV open reading frames (ORFs) 15 to 41 (IJkel *et al.*, 1999). This makes the engineering of SeMNPV for improved insecticidal activity or as expression vectors difficult to achieve. The generation of defective viruses in cell culture limits the structural and functional analysis of the SeMNPV genes and the isolation of recombinants with adequate infectivity *in vivo* and in cell culture.

SeMNPV has been isolated from many different geographical regions throughout the world (Vlak *et al.*, 1981; Gelernter & Federici, 1986*b*; Hara *et al.*, 1995*a*; Muñoz *et al.*, 1998). Wild-type (wt) SeMNPV isolates consisting of several genotypic variants are frequently found. This is typically indicated by the presence of submolar bands in restriction endonuclease digestion profiles of viral DNA (Muñoz *et al.*, 1998, 1999). Isolation of individual genotypic variants by *in vivo* cloning methods (Smith & Crook, 1988) has allowed the evaluation of the relative virulence of the different genotypic variants (Muñoz *et al.*, 1998, 1999). Since multiple passaging of SeMNPV in cultured insect cells results in the generation of defective viruses (Heldens *et al.*, 1996), cloning of genotypic variants of SeMNPV is difficult to obtain by conventional plaque purification techniques. Hence, a novel strategy was adopted in this study to generate genotypic variants of SeMNPV by cloning alternately *in vivo* and *in vitro*.

We previously reported that recombinants of *Autographa californica* (Ac) MNPV were successfully generated in *S. exigua* larvae by transfection of viral and transfer vector DNA into the haemocoel by lipofection (Hajós *et al.*, 1998). In this study we used a similar strategy to generate recombinants of SeMNPV. We also adopted a novel procedure to isolate SeMNPV recombinants by cloning alternately between *S. exigua* larvae and Se301 cultured cells to secure *in vivo* and *in vitro* infectivity. By this strategy an SeMNPV recombinant (SeXD1) was generated using green fluorescent protein (GFP) as a screening marker. This recombinant had a similar genetic make-up to one of the variants observed in the SeMNPV isolates and was able to replicate both *in vivo* and in cultured insect cells. However, it lacked 10·6 kbp of nucleotide sequence as compared to the complete SeMNPV genome. Bioassays indicated that the recombinant has superior speed of kill as compared to the wt isolate.

Methods

■ **Virus, insects and cells.** The SeMNPV-US1 isolate (Gelernter & Federici, 1986*b*) was originally obtained from B. A. Federici (Department of Entomology, University of California, Riverside, CA, USA) in the form of polyhedra and propagated in fourth instar *S. exigua* larvae (Smits *et al.*, 1988). Cultures of *S. exigua* were reared on an artificial diet at 27 °C, 70% humidity and a 16:8 h photoperiod. The *S. exigua* cell lines Se301 (Hara *et al.*, 1995*b*) and SeUCR (Gelernter & Federici, 1986*a*) were donated by T. Kawarabata (Institute of Biological Control, Kyushu University, Japan) and B. A. Federici, respectively. All cells were propagated at 27 °C in Grace's supplemented medium containing 10% foetal calf serum (FCS; Gibco). Viral DNA used for the generation of recombinant viruses and restriction endonuclease analysis was extracted from polyhedra produced in *S. exigua* larvae by standard methods (O'Reilly *et al.*, 1992).

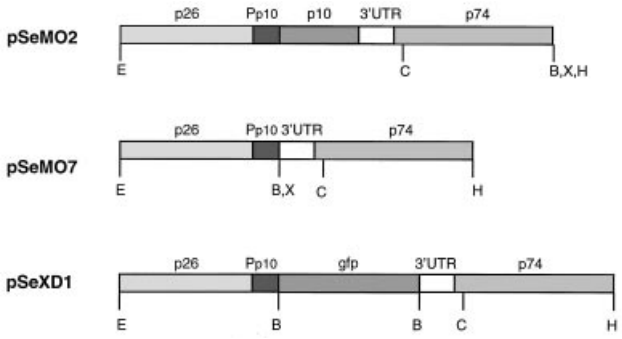

Fig. 1. Schematic representation of the *p10* locus in SeMNPV and in the *p10*-based transfer vectors pSeMO7 and pSeXD1. Plasmid pSeMO2 contains a 1448 bp *Eco*RI–*Bam*HI fragment derived from the *Xba*I H fragment of SeMNPV containing the *p10* locus and its flanking sequences. Plasmid pSeMO7 is the empty SeMNPV *p10* promoter-based transfer vector. In plasmid pSeXD1 the ORF for the green fluorescent protein (gfp) is present downstream of the *p10* promoter. Pp10, *p10* promoter; 3′UTR, *p10* 3′ untranslated region including a poly(A) motif; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I.

■ **Construction of an SeMNPV *p10* promoter-based transfer vector for GFP expression.** The SeMNPV 5·1 kbp *Xba*I H fragment containing the *p10* gene flanked by *p26* and *p74* sequences (Zuidema *et al.*, 1993; IJkel *et al.*, 1999) was used as a basis for the construction of an SeMNPV *p10* promoter-based transfer vector (Fig. 1). A 1448 bp *Eco*RI–*Bam*HI fragment was derived from the *Xba*I H fragment and cloned into pUC19 (pSeMO2). The *Bam*HI and *Xba*I sites located at one end of the insert were both removed by filling in with *Klenow*, resulting in plasmid pSeMO4. The 5′ flanking sequence of the SeMNPV *p10* locus, containing the 3′ end of the *p26* gene and the *p10* promoter, was isolated by PCR with the forward primer M13 and a specific antisense primer (5′ TCTAGACCTAAGGGATCCTAATGTATAATATAATTAC 3′) using pSeMO4 as template. With this PCR a *Bam*HI site was introduced immediately downstream of the adenosine residue of the *p10* translational start codon. The PCR product was cloned into pUC19 as a 513 bp *Eco*RI–*Bam*HI fragment (pSeMO5) and its identity was verified by sequence analysis. A second PCR was performed on pSeMO4 with the reverse M13 primer and a sense primer (5′ GGATCCCTTAGG-TCTAGATAAAACTTAACGACGACG 3′) to generate the 3′ flanking region of the transfer vector containing the *p10* 3′ untranslated region and the 3′ end of the *p74* gene. With this PCR an *Xba*I site was generated immediately upstream of the *p10* translational stop codon TAA. The PCR product was cloned into pUC19 as a 680 bp *Xba*I–*Hin*dIII fragment (pSeMO6). Sequence analysis showed the correct sequence between the introduced *Xba*I site and the internal *Cla*I site. A three-point ligation was performed to bring the 5′ and 3′ flanking regions of the *p10* gene together, separated by *Bam*HI and *Xba*I sites. An approximately 3·2 kbp *Eco*RI–*Cla*I fragment of pSeMO4, containing pUC19 sequences and part of the 3′ flanking sequence, was combined with the 513 bp *Eco*RI–*Bam*HI fragment of pSeMO5 and the 130 bp *Bam*HI–*Cla*I fragment of pSeMO6 to give pSeMO7. In this new vector the *Bam*HI site is juxtaposed to the

*Xba*I site. Finally, a 747 bp *Bam*HI fragment containing the GFP ORF derived from pUC19 GFP (Reiländer *et al*., 1996) was cloned into the *Bam*HI site of pSeMO7 to give the transfer vector pSeXD1.

■ **Generation of an SeMNPV *p10*⁻ recombinant expressing GFP.** An SeMNPV recombinant was generated by injection of viral and transfer vector DNA into the haemocoel of fourth instar *S. exigua* larvae according to Hajós *et al*. (1998) followed by alternate cloning between *S. exigua* larvae and Se301 cells. The injection into insect larvae was performed using a 1·5 ml volume B-D Pen (Becton & Dickinson) and 28-gauge half-inch NovoFine needles (Novo Nordisk). The injection solution was added to 1·5 ml injector cartridges (Eli Lilly) in a sterile hood (Hajós *et al*., 1998). Twenty µl of the cotransfection solution containing 0·4 µg circular SeMNPV DNA and 12 µg transfer vector pSeXD1 DNA, and 30% Cellfectin (Gibco-BRL) were injected into the haemocoel of each larva. Haemolymph was obtained from a cut proleg 3 days post-transfection and added to 5 ml of serum-free Grace's medium containing a few crystals of phenylthiourea, filtered through a 0·45 µm filter (Schleicher & Schuell) and stored at −80 °C. The haemolymph filtrate was tested for virus titre and the relative proportion of wt and recombinant SeMNPV by plaque assay determinations (O'Reilly *et al*., 1992). The assays were scored for fluorescence under a UV microscope.

Recombinant plaques were selected by their GFP expression and each plaque was diluted with 200 µl Grace's medium without FCS to elute extracellular virus. The virus was amplified in a 24-well plate by adding 100 µl of the plaque eluate to a well with approximately 2×10^4 Se301 cells. Wells with polyhedra-containing cells were harvested 3 days post-infection (p.i.) and the cells were suspended in 12 µl distilled water. *S. exigua* third instar larvae were then orally fed after adding the cell suspensions of each well onto *Chrysanthemum* leaf discs with a diameter of 4 mm and placed in 6-well tissue culture plates containing 1 ml 1·5% agarose layer to prevent desiccation. One larva was put in each well with one leaf disc. After consumption of the leaf disc (approx. 16 h) the larvae were placed on an artificial diet. Haemolymph was collected at 3 days p.i. from larvae showing infection symptoms (lethargy, impaired locomotion, pale appearance, no food consumption) and used to measure virus titre and to perform a second round of plaque purification. After three rounds of alternate *in vivo* and *in vitro* cloning, the SeMNPV recombinants were amplified in fourth instar *S. exigua* larvae.

■ **SDS–PAGE and Western blot analysis.** Se301 cells were infected at an m.o.i. of 10 with wt SeMNPV and the recombinant (SeXD1), respectively. Infected cells were harvested at 48 h p.i. and the proteins were analysed by electrophoresis in a 12·5% SDS–polyacrylamide gel using a Bio-Rad Mini-Protein II apparatus. Western blot analysis was performed with a GFP antibody (Molecular Probes) (1:2000 diluted) by standard methods (Sambrook *et al*., 1989).

■ **PCR, cloning and sequencing.** To analyse deletions in the SeMNPV *Pst*I D fragment, a PCR was performed with the Expand Long Template PCR system (Boehringer Mannheim) using forward primer A (5′ GTAGGGGACGCGAATTTGACTGTTGTTGCAG 3′) and reverse primer B (5′ CGCACGCTCCACGCTACTCGACTTTGATA 3′), corresponding to nt 17874 to 17904 and 29135 to 29163 of the SeMNPV genome (IJkel *et al*., 1999), respectively. The PCR products were cloned into pGEM-T (Promega) and sequence reactions were performed at the Sequencing Core Facility of Eurogentec using universal primers.

■ **Bioassays.** The infectivities of wt SeMNPV and recombinant SeXD1 were determined in a leaf disc bioassay as described by Bianchi *et al*. (2000). *Chrysanthemum* leaf discs were prepared using a cork borer with a diameter of 9 mm and placed individually in a 12-well tissue culture plate containing 1 ml 1·5% agarose. Droplets (3 µl) of polyhedra suspensions containing 0 (control), 3×10^3, 10^4, 3×10^4, 10^5, 3×10^5 polyhedra/ml were applied to each leaf disc and dried using a fan. One third instar *S. exigua* larva was added per well. For each dose 36 larvae were used. Larvae that consumed the whole leaf disc within 24 h were transferred to a 12-well tissue culture plate containing fresh artificial diet and were further reared at 27 °C. Mortality was recorded daily until all larvae had either pupated or died due to SeMNPV infection. The bioassay was performed in three repetitions.

The speed of action of wt SeMNPV and the recombinant SeXD1 was determined in a modified droplet-feeding bioassay (Hughes & Wood, 1981). Third instar *S. exigua* larvae were starved for 16 to 20 h at 27 °C prior to bioassaying. The larvae were allowed to drink from an aqueous suspension containing 10% (w/v) sucrose, 0·001% (w/v) SÄURE-blue and polyhedra at concentrations of 0 (control), 10^3, 3×10^3, 10^4, 3×10^4, 10^5 polyhedra/ml. The first 36 larvae that drank from the solution within 10 min were transferred to individual wells of three 12-well tissue culture plates with a fresh artificial diet. Larvae were reared at 27 °C, and mortality was recorded every 12 h until all larvae had either pupated or died. The bioassay was performed in four repetitions. Dose–mortality data were analysed with the computer program POLO (Russell *et al*., 1977). For the calculation of LD_{50} values, median ingested volumes of 0·55 µl for third instar *S. exigua* larvae were used, as measured by Bianchi *et al*. (2000). Median survival times (ST_{50}) were calculated using the Vistat program (version 2.1; Boyce Thompson Institute, Cornell University, Ithaca, NY, USA). Log LD_{50} and ST_{50} values were analysed by regression analysis and *t*-tests of pairwise differences between treatments with Genstat (Payne *et al*., 1993).

Results

Generation of *p10*⁻ recombinant SeXD1

To generate a *p10*⁻ SeMNPV recombinant, the transfer vector pSeXD1 carrying a GFP marker gene was constructed (Fig. 1). The size of transfer vector pSeXD1 was 4·6 kbp, and it contained 503 bp upstream (including the *p10* promoter) and 673 bp downstream [including the *p10* poly(A) motif; van Oers *et al*., 1999] of the SeMNPV *p10* ORF, and the 747 bp GFP gene driven by the authentic *p10* promoter.

Seventeen fourth instar larvae were injected with wt SeMNPV and pSeXD1 DNA at a ratio of 1:30 µg, corresponding to a molar ratio of approximately 1:800. Sixteen larvae survived the injection treatment. The haemolymph from these 16 larvae was transferred to 5 ml of Grace's medium without FCS. The controls included larvae injected with only viral DNA, only transfer vector DNA, only Cellfectin and untreated larvae. Plaque assays indicated that the total virus titre of the haemolymph was $3{\cdot}7 \times 10^4$ p.f.u./ml. The percentage of recombinants was approximately 3·3%, in agreement with data obtained previously for AcMNPV (Hajós *et al*., 1998). The GFP gene driven by the *p10* promoter of SeMNPV induced bright fluorescence, as observed with a UV microscope. With the help of GFP, it was easy to screen by fluorescence and pick recombinant plaques from Se301 cells. Several recombinant viruses were isolated by three rounds of alternate cloning between third instar *S. exigua* larvae and Se301 cells (see Methods). Finally, recombinant SeXD1 was amplified in fourth instar *S. exigua* larvae and analysed.

To confirm the location of the GFP gene insertion, recombinant SeXD1 DNA was examined by *Spe*I restriction

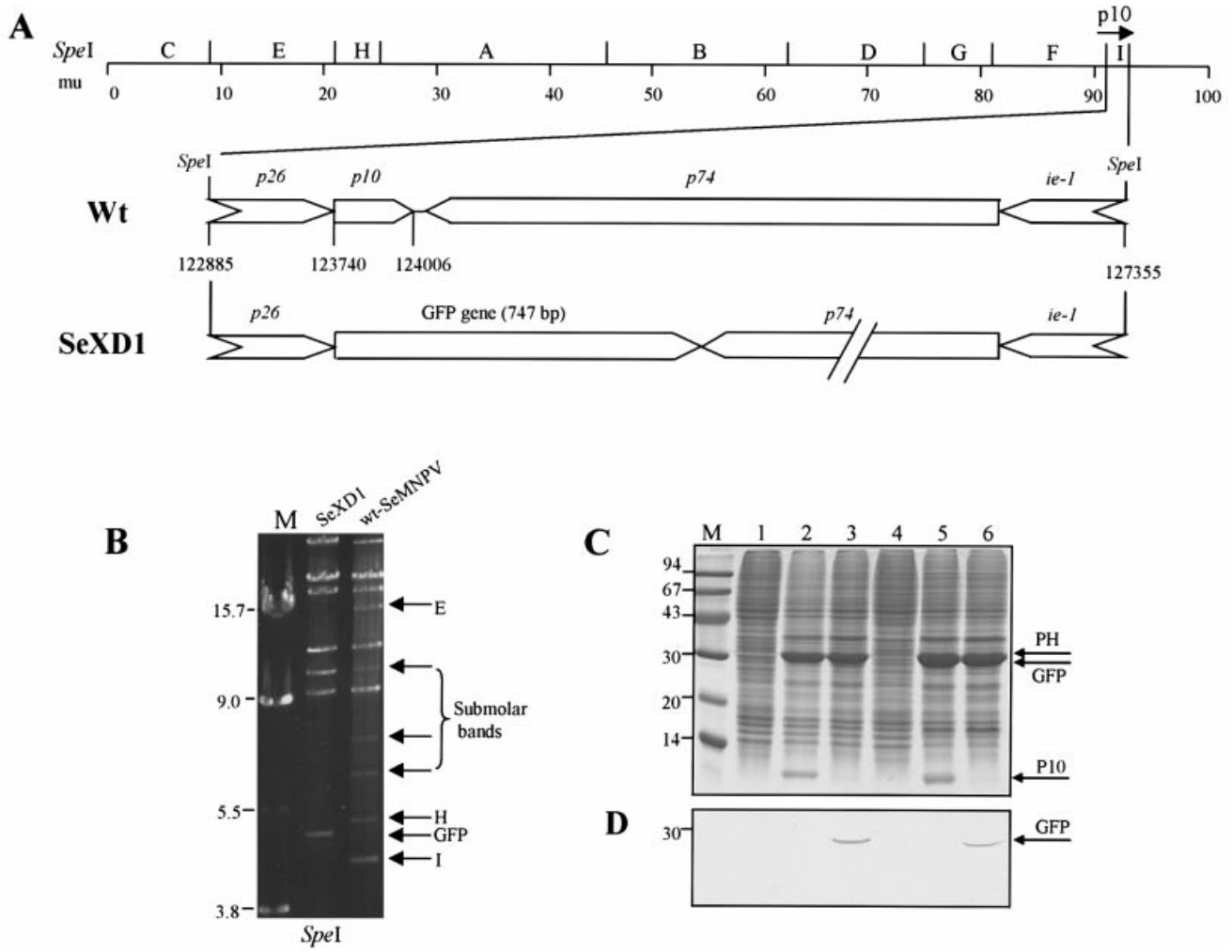

Fig. 2. Construction and analysis of SeXD1, a *p10*⁻ SeMNPV recombinant expressing GFP. (A) Construction of SeXD1. The top line represents the physical map of the wt SeMNPV genome for *Spe*I restriction endonuclease. *p10* is located in the 4·5 kb *Spe*I I fragment (corresponding to nt 122885 to 127355). The 264 bp *p10* ORF (corresponding to nt 123740 to 124006) was replaced with the 747 bp GFP gene; the *Spe*I I fragment became 5·0 kb in SeXD1. (B) *Spe*I restriction endonuclease analysis of genomic DNAs from wt SeMNPV and SeXD1. (C) and (D) SDS–PAGE and Western blot analysis using anti-GFP antibodies. Uninfected Se301 cells (lane 1), Se301 cells infected with wt SeMNPV (lane 2) and with SeXD1 (lane 3), uninfected SeUCR cells (lane 4), SeUCR cells infected with wt SeMNPV (lane 5) and with SeXD1 (lane 6). PH, polyhedrin.

endonuclease digestion. The SeMNPV *p10* gene is located on the 4·5 kbp *Spe*I I fragment corresponding to nt 122885 to 127355 of the genome (Fig. 2A) (IJkel *et al.*, 1999). When the 264 bp *p10* ORF (corresponding to nt 123740 to 124006) is replaced with the 747 bp GFP gene ORF, the *Spe*I I fragment would become 5·0 kbp (Fig. 2A). As shown in Fig. 2(B), the *Spe*I restriction endonuclease pattern of SeXD1 confirmed the insertion of the GFP ORF into the *p10* locus in SeXD1. SDS–PAGE and Western blot analysis showed the absence of the P10 protein, while GFP was expressed in both Se301 and SeUCR cells infected with SeXD1 (Fig. 2C and D, lanes 3 and 6).

Uninfected Se301 cells are shown in Fig. 3(A). At 16 h p.i., polyhedra were observed in about 20% of the Se301 cells infected with either wt SeMNPV or SeXD1 at an m.o.i. of 10 (data not shown). At 48 h p.i., polyhedra were observed in about 90% of the Se301 cells infected with wt SeMNPV (Fig. 3B) and in almost 100% of Se301 cells infected with SeXD1 (Fig. 3C). Bright fluorescence was observed in SeXD1-infected Se301 cells under the UV microscope (Fig. 3D). No fluorescence was observed either in wt SeMNPV-infected or in uninfected Se301 cells (data not shown).

Thus, UV microscopy, restriction enzyme analysis, SDS–PAGE and Western blot analysis demonstrated that the recombinant virus SeXD1 lacked the *p10* gene and expressed GFP. This recombinant was able to complete its replication cycle both in *S. exigua* larvae and in the cultured cell lines Se301 and SeUCR.

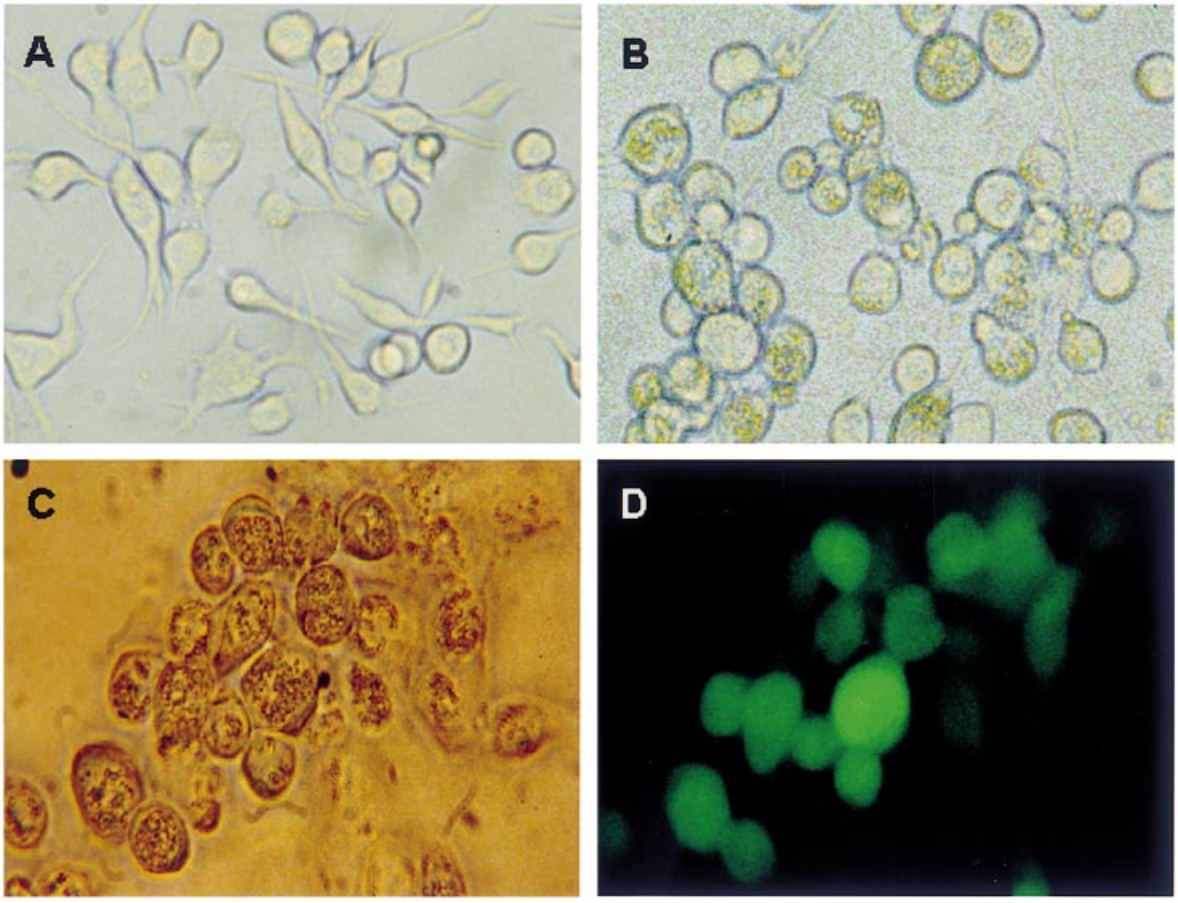

Fig. 3. Phase-contrast and UV micrographs of the *S. exigua* cell line Se301. Se301 cells (A) were infected with wt SeMNPV (B) and with SeXD1 (C and D) at 48 h p.i. Polyhedra were observed in phage contrast images of wt SeMNPV (B) and SeXD1-infected cells (C). The expression of GFP in SeXD1-infected cells is shown by irradiation with UV light (D).

Analysis of deletion mutants

Wt SeMNPV is made up of several genotypic variants (Muñoz *et al.*, 1998, 1999) and replication of SeMNPV in cultured cells often results in the generation of deletion mutants (Heldens *et al.*, 1996). To determine whether the recombinant SeXD1 is one of these variants, SeXD1 as well as wt SeMNPV were analysed with restriction endonucleases. The *Spe*I and *Pst*I digestions showed several submolar bands in wt SeMNPV (Figs 2B and 4B), indicating that the wt SeMNPV isolate is a mixture of genotypes. No submolar bands were found in the *Spe*I and *Pst*I digestion patterns of SeXD1 DNA (Figs 2B and 4B). However, the *Pst*I D, *Spe*I E and *Spe*I H fragments were absent in SeXD1 (Figs 2B and 4B), suggesting that although SeXD1 is genetically homogeneous it might be a deletion mutant. One of the submolar bands found after *Spe*I digestion of wt SeMNPV (Fig. 2B) is a molar band in the recombinant, suggesting that a variant with a similar deletion is present in wt SeMNPV.

To determine in more detail which region was absent, both SeXD1 and wt SeMNPV DNA were examined by PCR amplification. The restriction analysis had shown the absence of the *Pst*I D fragment in SeXD1, while the neighbouring fragments L and C were retained (Fig. 4A, B). Therefore, PCR primers were designed annealing approximately 100 bp up- and downstream of the *Pst*I D fragment in fragments L and C, respectively (see Methods). In an amplification from complete genomic SeMNPV DNA the PCR product should be 11289 bp, and a product of this size was indeed observed (Fig. 4A). Amplification from SeXD1 DNA, however, resulted in a single approximately 700 bp product (Fig. 4C), suggesting that about 10 kbp was deleted from SeXD1. PCR analysis also indicated that SeXD1 most likely contained a single genotype. Amplification using wt SeMNPV DNA as template resulted in at least five products, including 11 kbp, 2·8 kbp, 2·0 kbp, 1·2 kbp and 700 bp products (approximate sizes; Fig. 4C). These results suggested that the wt SeMNPV is a mixture containing several deletion mutant variants in this locus. Conclusions about the relative amounts of the variants cannot be drawn from this analysis, however, since smaller fragments are likely to be amplified more efficiently than larger ones.

The approximately 700 bp product was observed in both SeXD1 and wt SeMNPV (Fig. 4C), implying that SeXD1 might have originated from one particular genotypic variant in the wt

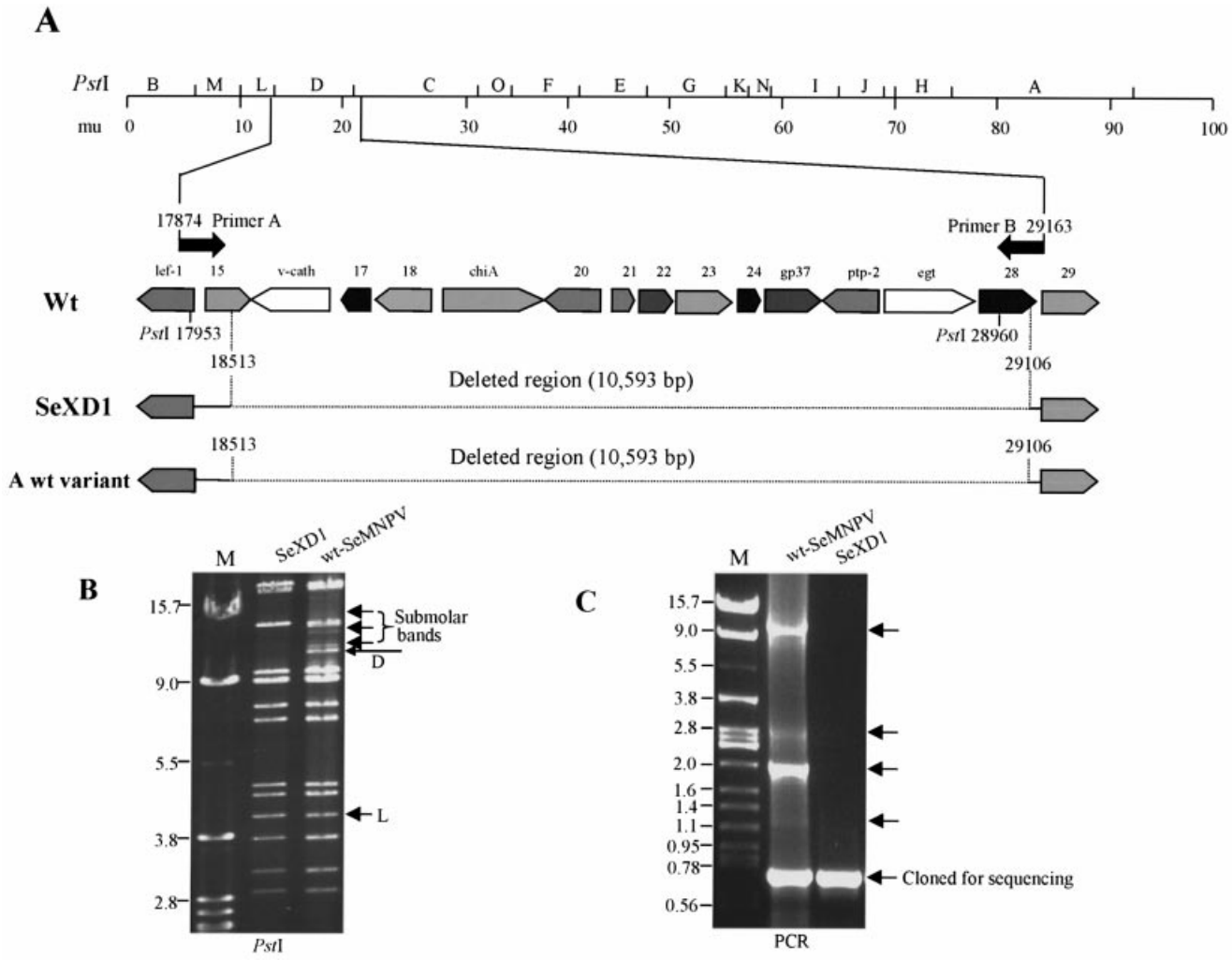

Fig. 4. Analysis of the deletion mutants. (A) The top line represents the physical map of the wt SeMNPV genome for *Pst*I restriction endonuclease. A 10593 bp fragment, including *egt*, *v-cath*, *gp37*, *chiA*, etc. (corresponding to nt 18513 to 29106; dashed horizontal line), was deleted in the recombinant SeXD1. The deletion in a genotypic variant of wt SeMNPV was also from nt 18513 to nt 29106 (a total of 10593 bp). (B) *Pst*I restriction endonuclease analysis of genomic DNAs from wt SeMNPV and SeXD1. The *Pst*I D fragment was absent in SeXD1. (C) PCR analysis of genomic DNAs from wt SeMNPV and SeXD1. The primers A and B correspond to nt 17874 to 17904 and 29135 to 29163, respectively.

SeMNPV isolate. To exactly locate the deleted region and to compare SeXD1 with wt SeMNPV, the approximately 700 bp fragments from both SeXD1 and wt SeMNPV were cloned into pGEM-T and sequenced. Sequence analysis showed the presence of both primers in the PCR products and mapped the deletion of SeXD1 from 13·7 to 21·6 m.u. (10593 bp, from nt 18513 to nt 29106) (Fig. 4A). The deletion in a genotypic variant of wt SeMNPV was also from nt 18513 to nt 29106, a total of 10593 bp (Fig. 4A). A total of 12 ORFs were completely deleted, encompassing SeMNPV ORFs 16 to 27 and including *ecdysteroid UDP glucosyl transferase* (*egt*), *gp37*, *chitinase* (*chiA*), *cathepsin* (*v-cath*), *ptp-2* and nine others. Two ORFs, ORF 15 and 28, were partially deleted. Therefore, the sequences maintained in SeXD1 and in one of the wt SeMNPV variants were the same, suggesting that SeXD1 is derived from an existing genotypic variant of wt SeMNPV.

SeXD1 was passaged in Se301 cells several times when purified but still retained the same deletion as its parental wt SeMNPV. The result indicated that the genotypic variant with a deletion of 10593 bp was quite stable. The result also indicated that naturally *egt*-deleted, *gp37*-deleted, *chiA*-deleted and *v-cath*-deleted genotypes existed in the wt SeMNPV population and that none of the deleted genes are required for viral DNA replication either *in vivo* or *in vitro*.

Biological activity and symptomatology of virus-infected *S. exigua* larvae

The insecticidal activities of the recombinant SeXD1 and wt SeMNPV were determined for third instar *S. exigua* larvae in terms of LD_{50} and ST_{50} (Table 1). The ST_{50} value of SeXD1 (70·2 h) was 25% lower than that of wt SeMNPV (93·1 h). The ST_{50} value was significantly different ($P < 0{\cdot}05$). The slopes of

Table 1. Dose–mortality (LD_{50}) and lethal time–mortality (ST_{50}) of wt SeMNPV and recombinant SeXD1 for third instar *S. exigua* larvae

The data in the table came from the statistical analysis. The LD_{50} was determined in three repetitions by a leaf disc bioassay and the ST_{50} in four repetitions by a droplet-feeding bioassay.

Viruses	Log LD_{50}	LD_{50} (OBs/larva)	Slope	ST_{50} (h)	Slope
wt SeMNPV	$4{\cdot}83^a \pm 0{\cdot}68$	125^a	$1{\cdot}50^a \pm 0{\cdot}32$	$93{\cdot}1^b \pm 5{\cdot}9$	$10{\cdot}92^a \pm 3{\cdot}86$
SeXD1	$6{\cdot}00^a \pm 1{\cdot}15$	403^a	$1{\cdot}26^a \pm 0{\cdot}34$	$70{\cdot}2^b \pm 6{\cdot}7$	$9{\cdot}17^a \pm 2{\cdot}16$

a, No significant difference; *b*, significantly different.

the filled time–mortality relationships were not significantly different for both viruses.

The LD_{50} value of SeXD1 [403 occlusion bodies (OBs)/larva] was approximately three times higher than that of wt SeMNPV (125 OBs/larva), but this was not significantly different ($P = 0{\cdot}094$) (Table 1). The slopes of the filled dose–mortality curves were not significantly different ($P = 0{\cdot}05$).

There were some differences in symptoms of wt SeMNPV and SeXD1-infected *S. exigua* larvae. The larvae infected with wt SeMNPV became pale and creamy in colour prior to death. After death infected insects rapidly liquefied. A small proportion of the wt SeMNPV-infected larvae turned black before liquefaction. The larvae infected with SeXD1 also became pale prior to death but all larvae turned black. In addition, the SeXD1-infected larvae did not liquefy after death and remained physically intact (data not shown), a typical phenotype of infection with a baculovirus lacking cathepsin and/or chitinase (Slack *et al.*, 1995; Hawtin *et al.*, 1997).

Discussion

Replication of SeMNPV in cultured cells results in the generation of deletion mutants which are not infectious to *S. exigua* larvae (Heldens *et al.*, 1996). This is the major reason why engineering of SeMNPV has been difficult to achieve in the past several years. Based on the successful generation of AcMNPV recombinants by cotransfection of viral and transfer vector DNA into the haemocoel of *S. exigua* larvae (Hajós *et al.*, 1998), and the supposition that a few intact SeMNPV would survive one or two passages in cultured cells, we adopted a procedure to engineer SeMNPV by alternate cloning between insect larvae and cultured cells. When the molar ratio between viral DNA and transfer vector was 1:30, recombinants were observed at 3·3%. This is in the same order of magnitude as in the case of AcMNPV, where approximately 2% has been recorded (Hajós *et al.*, 1998). Although the same amount of viral DNA per larva (0·4 μg) was used in the injection, the total virus titre in the haemolymph of the cotransfected larvae is much lower ($3{\cdot}7 \times 10^4$ p.f.u./ml) than found for AcMNPV ($5{\cdot}2 \times 10^8$ p.f.u./ml) (Hajós *et al.*, 1998). The result suggests that the transfection with SeMNPV DNA is less efficient than with AcMNPV DNA, but that the relative proportion of recombinants is more or less similar.

A wt SeMNPV isolate is made up of several genotypic variants; some of these contain large deletions and are helper-dependent (Muñoz *et al.*, 1998, 1999). PCR and sequence analysis showed that in the recombinant SeXD1 10593 bp of the SeMNPV sequence was deleted (Fig. 4A, C). The same procedure revealed the presence of a genotype with a deletion of the same size in wt SeMNPV (Fig. 4C). The question is whether the SeMNPV deletion naturally exists in the wt SeMNPV population or results from the passages in cultured cells. Since it was reported that extensive deletions in the SeMNPV genome occurred very quickly in the SeUCR cell line (Heldens *et al.*, 1996), it was generally thought that SeMNPV would loose its pathogenic effect *in vivo* after just one passage with multiple replication cycles in cultured cells and that it would be difficult to obtain SeMNPV variants that retained biological activity *in vivo* from cultured cells. However, with this novel approach we successfully selected several SeMNPV recombinants infectious *in vivo* and *in vitro*, one of which, SeXD1, was analysed in detail.

Restriction endonuclease and PCR analysis showed the presence of several other genotypes in wt SeMNPV (Figs 2B and 4B, C). After the first round of plaque purification using Se301 cells and the haemolymph of cotransfected larvae, we observed several plaques containing polyhedra that were not infectious for *S. exigua* larvae (X. Dai, unpublished data). However, most plaques were pathogenic for *S. exigua* larvae. In our study we picked plaques in Se301 cells 3 days p.i. and then amplified the plaques in Se301 cells for another 3 days before harvesting the polyhedra-containing cells. Thus, recombinant SeXD1 grown in Se301 cells for about two passages still retained its biological activity and consisted of a single genotype. Apparently in Se301 cells the deletion in SeMNPV does not happen as quickly as in SeUCR cells. Hara *et al.* (1993) reported that SeMNPV produced in Se301 cells was still

infectious for larvae. Recently, Choi *et al.* (1999) generated an SeMNPV polyhedrin$^-$ recombinant in these cells, but its infectivity for insects and its genetic make-up was not studied. Hence, there might be differences in the induction of defective viruses of SeMNPV between Se301 and SeUCR cells and some cell factors might be involved in the generation of deletion mutants.

SeXD1 lacked the *p10* gene of SeMNPV and expressed GFP. SeXD1 also lacked 10593 bp of additional sequence information of SeMNPV, including *egt*, *gp37*, *chiA*, *v-cath* and ten other genes located in this region (IJkel *et al.*, 1999). Bioassays showed that the ST_{50} value of SeXD1 was 25% lower than that of wt SeMNPV, but that the LD_{50} value of SeXD1 was approximately the same as for wt SeMNPV (Table 1). The result suggests that the absence of one or more genes may be responsible for the enhanced speed of kill. Various studies showed that deletion of *p10* did not lead to an increased speed of kill (Martens *et al.*, 1995; Bianchi *et al.*, 2000). Recent results also indicated that GFP does not affect the biological activity of *Helicoverpa armigera* SNPV (Chen *et al.*, 2000). It has been reported that the ecdysteroid *egt* is a key enzyme in abrogating the regulation of host insect metamorphosis (O'Reilly & Miller, 1989). It conjugates ecdysteroids with sugars and hence blocks moulting of the insect. Insects infected with an *egt*-deleted virus exhibit reduced feeding and earlier mortality compared to wt virus-infected larvae (O'Reilly & Miller, 1991; O'Reilly, 1995; Flipsen *et al.*, 1995). Another study has shown that the LT_{50} value of *egt*-deleted *Lymantria dispar* (Ld) MNPV was about 33% lower than that of wt LdMNPV for fifth instar *L. dispar* larvae (Slavicek *et al.*, 1999). Our findings are thus consistent with these studies on *egt* deletion mutants.

Of those ORFs deleted from SeXD1, ORFs 17, 18 and 21 have homologues in *Xestia c-nigrum* granulovirus (Hayakawa *et al.*, 1999). ORFs 15 and 28 have homologues in LdMNPV (Kuzio *et al.*, 1999). ORFs 20, 22, 23 and 24 are unique to SeMNPV (IJkel *et al.*, 1999), but their function is unknown. SeXD1 was able to replicate in *S. exigua* larvae as well as in the cultured Se301 and SeUCR cells, so all the deleted genes are dispensable for virus replication both *in vivo* and *in vitro*.

Baculovirus *gp37* encodes a spindle-like protein, clearly related to fusolin of entomopoxviruses (EPVs) (Dall *et al.*, 1993; Liu & Carstens, 1996; Mitsuhashi *et al.*, 1997). There is accumulating evidence that fusolin of EPVs can enhance NPV infection in insects (Mitsuhashi *et al.*, 1998; Hayakawa *et al.*, 1996). Baculovirus *gp37* might also be involved in enhancing virus infection in insects (Phanis *et al.*, 1999) and the *gp37*/*fusolin* gene family might be essential for virus replication (Wu & Miller, 1989). In the present study, the absence of *gp37* did not affect virus replication in a detectable way either in cell culture or in insects. Thus, it remains enigmatic what the function of *gp37* is in the biology of baculovirus infection.

The baculovirus-infected insect host liquefies after death (Volkman & Keddie, 1990) and polyhedra are released. This process plays an important role in ensuring the efficient dissemination of virus by physical forces such as wind and rain splash. It has been reported that *chiA* and *v-cath* are involved in the liquefaction process of virus-infected insect larvae (Ohkawa *et al.*, 1994; Rawlings *et al.*, 1992; Slack *et al.*, 1995; Hawtin *et al.*, 1997). Recombinant SeXD1 with a *chiA* and *v-cath* deletion could not liquefy *S. exigua* larvae, consistent with previous reports. Gopalakrishnan *et al.* (1995) reported that a recombinant AcMNPV containing a *Manduca sexta chiA* gene required less time to kill *Spodoptera frugiperda* fourth instar larvae when injected into the haemocoel. However, Hawtin *et al.* (1997) reported that deletion of *chiA* or *v-cath* from AcMNPV had no significant effect on LD_{50} or ST_{50} of the recombinant. It is not clear whether the absence of *chiA* and *v-cath* has any effect on the LD_{50} value of SeXD1.

As a result of fluorescence microscopic studies using GFP as a marker, we observed that upon cotransfection of insect larvae SeMNPV recombination took place predominantly in fat body cells. In contrast, with AcMNPV, the recombination upon cotransfection was found to take place typically in the haemocytes (data not shown). GFP also proved to be a helpful marker in the screening of SeMNPV recombinants. This marker will be useful in analysing the pathological effects of this virus in target and non-target hosts using, for example, confocal laser scanning microscopy. The procedure to generate recombinant viruses followed in this paper is applicable to many baculovirus species, for instance, to generate recombinants with improved insecticidal characteristics. The method applied in this paper may also be useful for the investigation of naturally occurring genotypic variants in virus isolates and their insecticidal properties. The isolation of SeXD1 confirms a previous observation by *in vivo* cloning of SeMNPV (Muñoz *et al.*, 1998, 1999) that genotypes with different biological and insecticidal properties exist in natural baculovirus isolates.

The authors thank Magda Usmany for assistance with plaque purification, Felix Bianchi for assistance with the statistical analysis of the bioassay data, Jan van Lent for supplying GFP antibody, Xinwen Chen and Martin Erlandson for their useful suggestions. This research was supported in part by the Dutch–Israeli Agricultural Research Program (DIARP) 97/29, by the National Natural Science Foundation of China 39730030, and by a Corporate European 'Aid to Education' grant from E.I. DuPont and Nemours & Co.

References

Bianchi, F. J., Snoeijing, I., Van der Werf, W., Mans, R. M., Smits, P. H. & Vlak, J. M. (2000). Biological activity of SeMNPV, AcMNPV, and three AcMNPV deletion mutants against *Spodoptera exigua* larvae (Lepidoptera: Noctuidae). *Journal of Invertebrate Pathology* **75**, 28–35.

Chen, X., Sun, X., Hu, Z. H., Li, M., O'Reilly, D. R., Zuidema, D. & Vlak, J. M. (2000). Genetic engineering of *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus as an improved pesticide. *Journal of Invertebrate Pathology* (in press).

Choi, J. Y., Woo, S. D., Je, Y. H. & Kang, S. K. (1999). Development of a novel expression vector system using *Spodoptera exigua* nucleopolyhedrovirus. *Molecules and Cells* **9**, 504–509.

Dall, D., Sriskantha, A., Vera, A., Lai-Fook, J. & Symonds, T. (1993). A gene encoding a highly expressed spindle body protein of *Heliothis armigera* entomopoxvirus. *Journal of General Virology* **74**, 1811–1818.

Flipsen, J. T., Mans, R. M., Kleefsman, A. W., Knebel-Mörsdorf, D. & Vlak, J. M. (1995). Deletion of the baculovirus ecdysteroid UDP-glucosyltransferase gene induces early degeneration of Malpighian tubules in infected insects. *Journal of Virology* **69**, 4529–4532.

Gelernter, E. D. & Federici, B. (1986*a*). Continuous cell line from *Spodoptera exigua* (Lepidoptera: Noctuidae) that supports replication of nuclear polyhedrosis viruses from *Spodoptera exigua* and *Autographa californica*. *Journal of Invertebrate Pathology* **48**, 199–207.

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

Gopalakrishnan, B., Muthukrishnan, S. & Kramer, K. J. (1995). Baculovirus-mediated expression of a *Manduca sexta* chitinase gene: properties of the recombinant protein. *Insect Biochemistry and Molecular Biology* **25**, 255–265.

Hajós, J. P., Zuidema, D., Kulcsár, P., Heldens, J. G. M., Závodszky, P. & Vlak, J. M. (1998). Recombination of baculovirus DNA following lipofection of insect larvae. *Archives of Virology* **143**, 2045–2050.

Hara, K., Funakoshi, M., Tsuda, K. & Kawarabata, T. (1993). New *Spodoptera exigua* cell lines susceptible to *Spodoptera exigua* nuclear polyhedrosis virus. *In Vitro Cellular Developmental Biology* **29A**, 904–907.

Hara, K., Funakoshi, M. & Kawarabata, T. (1995*a*). *In vivo* and *in vitro* characterization of several isolates of *Spodoptera exigua* nuclear polyhedrosis virus. *Acta Virology* **39**, 215–222.

Hara, K., Funakoshi, M. & Kawarabata, T. (1995*b*). A cloned cell line of *Spodoptera exigua* has a highly increased susceptibility to the *Spodoptera exigua* nuclear polyhedrosis virus. *Canadian Journal of Microbiology* **4**, 1111–1116.

Hawtin, R. E., Zarkowska, T., Arnold, K., Thomas, C. J., Gooday, G. W., King, L. A., Kuzio, J. A. & Possee, R. D. (1997). Liquefaction of *Autographa californica* nucleopolyhedrovirus-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. *Virology* **238**, 243–253.

Hayakawa, T., Xu, J. & Hukuhara, T. (1996). Cloning and sequencing of the gene for an enhancing factor from *Pseudaletia separata* entomopoxvirus. *Gene* **177**, 269–270.

Hayakawa, T., Ko, R., Okano, K., Seong, S., Goto, C. & Maeda, S. (1999). Sequence analysis of the *Xestia c-nigrum* granulovirus genome. *Virology* **262**, 277–297.

Heldens, J. G. M., van Strien, E. A., Feldmann, A. M., Kulcsár, P., Munoz, D., Leisy, D. J., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1996). *Spodoptera exigua* multicapsid nucleopolyhedrovirus deletion mutants generated in cell culture lack virulence *in vivo*. *Journal of General Virology* **77**, 3127–3134.

Heldens, J. G. M., Liu, Y., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1997). Characterization of a putative *Spodoptera exigua* multicapsid nucleopolyhedrovirus helicase gene. *Journal of General Virology* **78**, 3101–3114.

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

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

Kuzio, J., Pearson, M. N., Harwood, S. H., Funk, C. J., Evans, J. T., Slavicek, J. M. & Rohrmann, G. F. (1999). Sequence and analysis of the genome of a baculovirus pathogenic for *Lymantria dispar*. *Virology* **253**, 17–34.

Liu, J. J. & Carstens, E. B. (1996). Identification, molecular cloning, and transcription analysis of the *Choristoneura fumiferana* nuclear polyhedrosis virus spindle-like protein gene. *Virology* **223**, 396–400.

Martens, J. W. M., van Oers, M. M., Van de Bilt, B., Vlak, J. M. & Oudshoorn, P. (1995). Development of a baculovirus vector that facilitates the generation of *p10*-based recombinants. *Journal of Virological Methods* **52**, 15–19.

Mitsuhashi, W., Saito, H. & Sato, M. (1997). Complete nucleotide sequence of the fusolin gene of an entomopoxvirus in the cupreous chafer, *Anomala cuprea* Hope (Coleoptera: Scarabeidae). *Insect Biochemistry and Molecular Biology* **27**, 869–876.

Mitsuhashi, W., Furuta, Y. & Sato, M. (1998). The spindles of an entomopoxvirus of coleoptera (*Anomala cuprea*) strongly enhance the infectivity of a nucleopolyhedrovirus in lepidoptera. *Journal of Invertebrate Pathology* **71**, 186–188.

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

Muñoz, D., Murillo, R., Krell, P. J., Vlak, J. M. & Caballero, P. (1999). Four genotypic variants of a *Spodoptera exigua* nucleopolyhedrovirus (SeSP2) are distinguishable by a hypervariable genomic region. *Virus Research* **59**, 61–74.

Ohkawa, T., Majima, M. & Maeda, S. (1994). A cysteine proteinase encoded by the baculovirus *Bombyx mori* nuclear polyhedrovirus. *Journal of Virology* **68**, 6619–6625.

O'Reilly, D. R. (1995). Baculovirus-encoded ecdysteroid UDP-glucosyltransferase. *Insect Biochemistry and Molecular Biology* **26**, 541–550.

O'Reilly, D. R. & Miller, L. K. (1989). A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyltransferase. *Science* **245**, 1110–1112.

O'Reilly, D. R. & Miller, L. K. (1991). Improvement of a baculovirus pesticide by deletion of the *egt* gene. *Bio/Technology* **9**, 1086–1089.

O'Reilly, D. R., Miller, L. K. & Luckow, V. A. (1992). *Baculovirus Expression Vectors: A Laboratory Manual*. New York: W. H. Freeman.

Payne, R. W., Lane, P. W., Digby, P. G. N., Harding, S. A., Leech, P. K., Morgan, G. W., Todd, A. D., Thompson, R., Tunnicliffe, W. G., Welham, S. J. & White, R. P. (1993). *Genstat 5 Release 3 Reference Manual*. Oxford, UK: Clarendon Press.

Phanis, C. G., Miller, D. P., Cassar, S. C., Tristem, M., Thiem, S. M. & O'Reilly, D. R. (1999). Identification and expression of two baculovirus *gp37* genes. *Journal of General Virology* **80**, 1823–1831.

Rawlings, N. D., Pearl, L. H. & Buttle, D. J. (1992). The baculovirus *Autographa californica* nuclear polyhedrosis virus genome includes a papain-like sequence. *Biological Chemistry Hoppe-Seyler* **373**, 1211–1215.

Reiländer, H., Haase, W. & Maul, G. (1996). Functional expression of the *Aequorea victoria* green fluorescent protein in insect cells using the baculovirus expression system. *Biochemical and Biophysical Research Communications* **219**, 14–20.

Russell, R. M., Robertson, J. L. & Savin, N. E. (1977). POLO: a new computer program for probit analysis. *Bulletin of the Entomological Society of America* **23**, 209–213.

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

Slack, J. M., Kuzio, J. & Faulkner, P. (1995). Characterization of *v-cath*, a cathepsin L-like proteinase expressed by the baculovirus *Autographa californica* multiple nuclear polyhedrosis virus. *Journal of General Virology* **76**, 1091–1098.

Slavicek, J. M., Popham, H. J. R. & Riegel, C. I. (1999). Deletion of the *Lymantria disper* multicapsid nucleopolyhedrovirus ecdystroid UDP-glucosyl transferase gene enhances viral killing speed in the last instar of the gypsy moth. *Biological Control* **16**, 91–103.

Smith, I. R. L. & Crook, N. E. (1988). *In vivo* isolation of baculovirus genotypes. *Virology* **166**, 240–244.

Smits, P. H., Van de Vrie, M. & Vlak, J. M. (1988). Nuclear polyhedrosis virus control of *Spodoptera exigua* on glasshouse crops. *Entomologia Experimentalis et Applicata* **43**, 73–80.

Smits, P. H. & Vlak, J. M. (1994). Registration of the first viral insecticide in the Netherlands: the development of Spod-X, based on *Spodoptera exigua* nuclear polyhedrosis virus. *Mededelingen van de Faculteit Landbouw-wetenschappen Rijksuniversiteit Gent* **59**, 385–392.

van Oers, M. M., Vlak, J. M., Voorma, H. O. & Thomas, A. A. M. (1999). Role of the 3′ untranslated region of baculovirus p10 mRNA in high-level expression of foreign genes. *Journal of General Virology* **80**, 2253–2262.

van Strien, E. A., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1992). Nucleotide sequence and transcriptional analysis of the polyhedrin gene of *Spodoptera exigua* nuclear polyhedrosis virus. *Journal of General Virology* **73**, 2813–2821.

van Strien, E. A., Jansen, B. J. H., Mans, R. M. W., Zuidema, D. & Vlak, J. M. (1996). Sequence and transcriptional analysis of the ubiquitin gene cluster in the genome of *Spodoptera exigua* nucleopolyhedrovirus. *Journal of General Virology* **77**, 2311–2319.

van Strien, E. A., Faktor, O., Hu, Z. H., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1997). Baculoviruses contain a gene for the large subunit of ribonucleotide reductase. *Journal of General Virology* **78**, 2365–2377.

Vlak, J. M., van Frankenhuyzen, K. & Peters, D. (1981). Identification of a new nuclear polyhedrosis virus from *Spodoptera exigua*. *Journal of Invertebrate Pathology* **38**, 297–298.

Volkman, L. E. & Keddie, B. A. (1990). Nuclear polyhedrosis virus pathogenesis. *Seminars in Virology* **1**, 249–254.

Wu, J. & Miller, L. K. (1989). Sequence, transcription and translation of a late gene of the *Autographa californica* nuclear polyhedrosis virus encoding a 34.8K polypeptide. *Journal of General Virology* **70**, 2449–2459.

Zuidema, D., van Oers, M. M., van Strien, E. A., Caballero, P. C., Klok, E.-J., Goldbach, R. W. & Vlak, J. M. (1993). Nucleotide sequence and transcriptional analysis of the p10 gene of *Spodoptera exigua* nuclear polyhedrosis virus. *Journal of General Virology* **74**, 1017–1024.

Received 17 March 2000; Accepted 19 June 2000
Published ahead of print (30 June 2000) in JGV Direct as
DOI 10.1099/vir.0.17048-0