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 Pang2 and Just M. Vlak1

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 beetle 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, 1986a), Se301 (Hara et al., 1995b) and IZD2109 (B. Möckel, personal communication), and susceptibility to SeMNPV has been reported (Hara et al., 1998).
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 (Jlkel 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 (Vlek et al., 1981; Gelernter & Federici, 1986b; Hara et al., 1995; 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 Spaecia 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 in vivo Spaecia 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 isolate.

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Methods

**Virus, insects and cells.** The SeMNPV-US1 isolate (Gelernter & Federici, 1986b) 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 Spaecia larvae (Smits et al., 1986). Cultures of Spaecia were reared on an artificial diet at 27 °C, 70% humidity and a 16:8 h photoperiod. The Spaecia cell lines Se301 (Hara et al., 1995b) and SeUCR (Gelernter & Federici, 1986a) 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 Spaecia larvae by standard methods (O’Reilly et al., 1992).

**Construction of an SeMNPV p10 promoter-based transfer vector for GFP expression.** The SeMNPV 51 kb Xbal H fragment containing the p10 gene flanked by p26 and p74 sequences (Zuidema et al., 1993; Jlkel et al., 1999) was used as a basis for the construction of an SeMNPV p10 promoter-based transfer vector (Fig. 1). A 1448 bp EcoRI–BanHI fragment was derived from the Xbal H fragment and cloned into pUC19 (pSeMO2). The BamHI and Xbal 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 5' end of the p26 gene and the p10 promoter, was isolated by PCR with the forward primer M13 and a specific antisense primer (5' TCTAGACCTAGGGATCTTAACTTAAATTATATTAC 3') using pSeMO4 as template. With this PCR a BamHI 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 EcoRI–BanHI 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' GGATCCCTAGG–TCTAGATAAAATCTTAAACGAGCGC 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 Xbal site was generated immediately upstream of the p10 translational stop codon TAA. The PCR product was cloned into pUC19 as a 680 bp Xbal–HindIII fragment (pSeMO6). Sequence analysis showed the correct sequence between the introduced Xbal site and the internal ClaI site. A three-point ligation was performed to bring the 5' and 3' flanking regions of the p10 gene together, separated by BamHI and Xbal sites. An approximately 3.2 kb EcoRI–ClaI fragment of pSeMO4, containing pUC19 sequences and part of the 3' flanking sequence, was combined with the 513 bp EcoRI–BanHI fragment of pSeMO5 and the 130 bp BamHI–ClaI fragment of pSeMO6 to give pSeMO7. In this new vector the BamHI site is juxtaposed to the
Isolation of an SeMNPV recombinant

Generation of an SeMNPV p10" recombinant expressing GFP. An SeMNPV recombinant was generated by injection of viral and transfer vector DNA into the haemocoele of fourth instar S. exigua larvae according to Hajois 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 (El Lilly) in a sterile hood (Hajois 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 haemocoele 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 x 10⁶ 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 a 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 pol1 D fragment, a PCR was performed with the Expand Long Template PCR system (Boehringer Mannheim) using forward primer A (5’ GTAGGGGACGCCAATTGAGCTGTGTGTATTG 3’) and reverse primer B (5’ CGACGGCTCAGGCTACTGATTTGATA 3’), corresponding to nt 17874 to 17904 and 29135 to 29163 of the SeMNPV genome (Ike 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 recombinantSeXD1 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 x 10⁶, 10⁷, 3 x 10⁷, 10⁸, 3 x 10⁸ 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% (v/v) sucrose, 0.01% (w/v) SAURE-blue and polyhedra at concentrations of 0 (control), 10⁶, 3 x 10⁷, 10⁸, 3 x 10⁹ 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 LD₅₀ 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 (ST₅₀) were calculated using the Vistat program (version 2.1; Boyce Thompson Institute, Cornell University, Ithaca, NY, USA). Log LD₅₀ and ST₅₀ 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 kb, 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 3.7 x 10⁸ p.f.u./ml. The percentage of recombinants was approximately 3.3%, in agreement with data obtained previously for AcMNPV (Hajois 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 SpeI restriction

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endonuclease digestion. The SeMNPV p10 gene is located on the 4·5 kbp SpeI 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 SpeI I fragment would become 5·0 kbp (Fig. 2A). As shown in Fig. 2(B), the SpeI 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.
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 SpeI and PstI 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 SpeI and PstI digestion patterns of SeXD1 DNA (Figs 2B and 4B). However, the PstI D, SpeI E and SpeI 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 SpeI 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 PstI 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 PstI D fragment in fragments L and C, respectively (see Methods). In an amplification from complete genomic SeMNPV DNA the PCR product should be 11,289 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
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’ to 21’ of 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). (B) PstI restriction endonuclease analysis of genomic DNAs from wt SeMNPV and SeXD1. The PstI 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.

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 LD50 and ST50 (Table 1). The ST50 value of SeXD1 (70.2 h) was 25% lower than that of wt SeMNPV (93.1 h). The ST50 value was significantly different (P < 0.05). The slopes of
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 ($P = 0.094$) (Table 1). The slopes of the filled dose–mortality curves were not significantly different ($P = 0.05$).

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 ($3.7 \times 10^4$ p.f.u./ml) than found for AcMNPV ($5.2 \times 10^8$ 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 lose 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

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**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.

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Log LD$_{50}$ (OBs/larva)</th>
<th>LD$_{50}$ (OBs/larva)</th>
<th>Slope</th>
<th>ST$_{50}$ (h)</th>
<th>Slope</th>
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<tr>
<td>wt SeMNPV</td>
<td>4.83$^a$ ± 0.68</td>
<td>125$^a$</td>
<td>1.50$^a$ ± 0.32</td>
<td>93-1$^b$ ± 5.9</td>
<td>10-92$^b$ ± 3.86</td>
</tr>
<tr>
<td>SeXD1</td>
<td>6.00$^a$ ± 1.15</td>
<td>403$^a$</td>
<td>1.20$^a$ ± 0.34</td>
<td>70-2$^b$ ± 6.7</td>
<td>9-17$^b$ ± 2.16</td>
</tr>
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$^a$ No significant difference; $^b$ significantly different,
LdMNPV for fifth instar (Ld) MNPV was about 33% lower than that of wt et al.

SeXD1 was able to replicate in cultured Se301 and SeUCR cells, so all the deleted genes are dispensable for virus replication both in vivo and in vitro. 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 ST50 value of SeXD1 was 25% lower than that of wt SeMNPV, but that the LD50 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 moultng 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 LT50 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 LD50 or ST50 of the recombinant. It is not clear whether the absence of chiA and v-cath has any effect on the LD50 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.

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