Journal of General Virology (1999), **80**, 1045–1053. Printed in Great Britain

Specificity of polyhedrin in the generation of baculovirus occlusion bodies

ZhiHong Hu,[1,2] Thomas Luijckx,[1] Leonie C. van Dinten,[1] Monique M. van Oers,[1] József P. Hajós,[1,3] Felix J. J. A. Bianchi,[1] Jan W. M. van Lent,[1] Douwe Zuidema[1] and Just M. Vlak[1]

[1] Department of Virology, Wageningen Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands
[2] Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, People's Republic of China
[3] Institute of Enzymology, Biological Research Centre, Hungarian Academy of Sciences, PO Box 7, 1518 Budapest, Hungary

The role of polyhedrin in the occlusion of virions was studied by substituting two heterologous polyhedrin-coding sequences, one from a multiple-nucleocapsid (M) nucleopolyhedrovirus (NPV) of *Spodoptera exigua* (Se) and one from a single-nucleocapsid (S) NPV of *Buzura suppressaria* (BusuNPV), into the genome of *Autographa californica* (Ac) MNPV. Both heterologous polyhedrin genes were highly expressed and polyhedra were produced in the nuclei of cells infected with the respective recombinant AcMNPVs. Polyhedra produced by the recombinant with BusuNPV polyhedrin showed normal occlusion of multiple-nucleocapsid virions and were equally as infectious to *S. exigua* larvae as were wild-type AcMNPV polyhedra. This indicates that virion occlusion is not specific with respect to whether the virions or polyhedrin are from an SNPV or MNPV. Polyhedra produced by the recombinant containing the SeMNPV polyhedrin had an altered morphology, being pyramidal rather than polyhedral in shape, and many fewer virions were occluded. These occlusion bodies were less infectious to *S. exigua* larvae than were those of wild-type AcMNPV. These results indicate that virion occlusion is a finely controlled process that is to some extent specific to the polyhedrin involved and may also require other viral or host factors for optimal morphogenesis.

Introduction

The baculoviruses are a large group of viruses that are pathogens for arthropod, mainly insect, hosts and that occlude their virions in large, proteinaceous capsules or occlusion bodies (OBs). The family *Baculoviridae* is composed of two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Murphy *et al.*, 1995). In GV, a single virion is occluded in an OB, whereas in NPV many virions are occluded. According to the number of nucleocapsids enveloped in virions, NPVs can be distinguished as single-nucleocapsid (S) NPVs or multiple-nucleocapsid (M) NPVs. The genetic basis for the difference in envelopment and occlusion strategy is unknown.

The baculovirus replicative cycle is biphasic, generating two distinct progeny phenotypes; the budded virus (BV), needed for the dissemination of infection within insect tissues, and the occlusion-derived virus (ODV), needed to spread the infection to other susceptible larvae. The function of the OB is most likely to protect the ODVs against physical and (bio)chemical decay and to allow the virions to retain their biological activity outside the host, even for many years. Furthermore, OBs might protect ODVs against proteolytic decay during the last stages of infection. OBs are surrounded by a carbohydrate-rich structure called the calyx (Minion *et al.*, 1979). The major protein component of the calyx is the polyhedron envelope protein, which seems to be associated with the carbohydrate residues of the calyx via a thiol–glycosidic linkage through cysteine residues (Whitt & Manning, 1988; Gombart *et al.*, 1989). The calyx is important in preventing polyhedron aggregation and in further protecting polyhedra from mechanical damage (Zuidema *et al.*, 1989; Gross *et al.*, 1994).

The factors directing the occlusion of ODVs into OBs are not well known. Polyhedrin and ODVs are likely to be the two main entities directly involved in ODV occlusion. Furthermore, it has been shown that a mutation in the 25K protein results in abnormal ODV envelopment, lack of viral occlusion and reduced OB formation (Harrison & Summers, 1995*a*, *b*). Since the 25K protein is not a component of ODVs and a mutation

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

0001-5930 © 1999 SGM

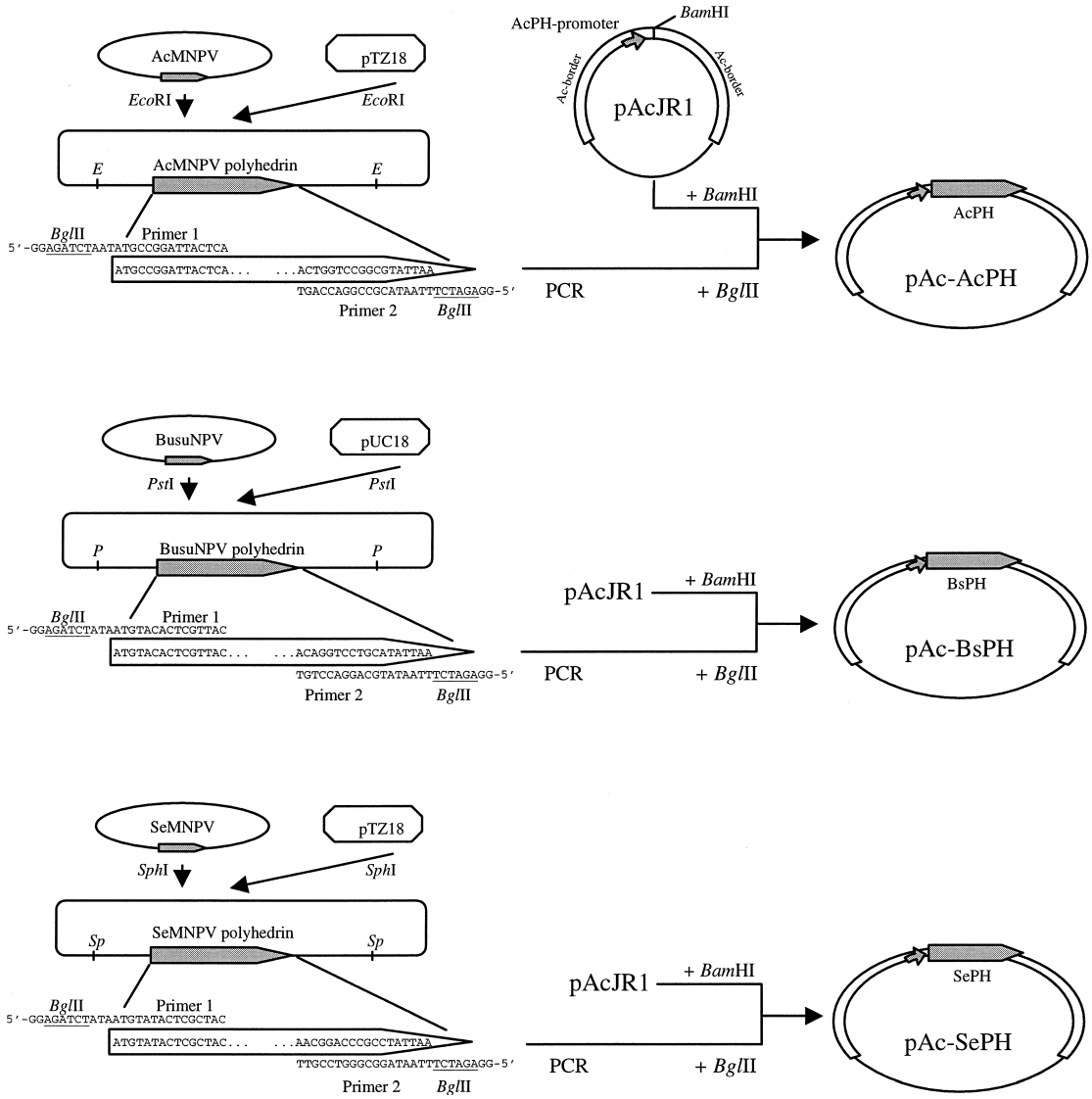

Fig. 1. Construction scheme for transfer vectors pAc-AcPH, pAc-BsPH and pAc-SePH, using parental AcMNPV transfer vector pAcJR1 and PCR products from the BusuNPV, SeMNPV and AcMNPV polyhedrin genes. The ORFs of the various polyhedrin genes were cloned downstream of the AcMNPV promoter. The primer sequences and restriction sites are indicated.

in the 25K gene resulted in reduced polyhedrin synthesis and nuclear localization (Jarvis *et al.*, 1992), the 25K protein may play an indirect role in the virion occlusion process (Harrison & Summers, 1995*a*, *b*).

Polyhedrin has been the subject of several reviews because of its unique properties and its pivotal role in the dissemination and survival of the virus (Vlak & Rohrmann, 1985; Rohrmann, 1986, 1992). So far, about 30 polyhedrin genes of different baculoviruses have been sequenced and they exhibit a highly conserved structure, with amino acid identity of at least 70% among lepidopteran NPVs. Research on the polyhedrin gene of *Autographa californica* (Ac) MNPV has revealed a nuclear localization signal (KRKK) located at amino acids 32–35 and a domain required for assembly into occlusion-like structures at amino acids 19–110 (Jarvis *et al.*, 1991). In contrast to the extensive sequence information, little is known about how polyhedrin plays its role in the occlusion process. For example, what determines the size and form of polyhedra, and are polyhedrins specific in their occlusion of single or multiple ODVs? Although polyhedrin is a highly conserved protein, it is not known whether all of its functional domains and recognition signals are universal to all NPVs.

In this study, we analysed the role of two polyhedrins, one from *Spodoptera exigua* (Se) MNPV (van Strien *et al.*, 1992) and the other from an SNPV, *Buzura suppressaria* (Busu) NPV (Hu *et al.*, 1993), in the occlusion process. The questions of whether an SNPV polyhedrin would occlude ODVs from an MNPV and whether other virus-specific factors are involved in the

occlusion process were addressed. To this end, recombinant AcMNPVs were constructed by replacing the AcMNPV polyhedrin-coding sequence with the homologous sequence of either BusuNPV or SeMNPV. As SeMNPV OBs are considerably smaller on average than AcMNPV, the study should also shed light on the extent to which the size and shape of OBs are determined by the polyhedrin itself or by other viral or host factors. The resulting recombinants were examined biochemically, by electron microscopy and in bioassays.

Methods

■ **Cells and viruses.** The *Spodoptera frugiperda* cell line IPLB-Sf21-AE (Sf21) (Vaughn *et al*., 1977) was maintained in TNM-FH medium (Hink, 1970) supplemented with 10 % foetal calf serum at 27 °C. The C6 strain of AcMNPV (Ayres *et al*., 1994) was used as a wild-type control; its polyhedrin-negative mutant BacPAK6 (Kitts & Possee, 1993) was used as the parental virus for construction of recombinants. Sf21 cells were infected at a multiplicity of 10 $TCID_{50}$ per cell, as described previously (van Oers *et al*., 1994).

■ **Generation of transfer vectors and recombinants.** The transfer vector pAc-BsPH, containing the polyhedrin gene of BusuNPV (Hu *et al*., 1993) in an AcMNPV transfer vector, was constructed as follows. The ORF was amplified by PCR from plasmid pBsPD containing the 7·8 kbp *Pst*I-D fragment of BusuNPV DNA (Hu *et al*., 1998). PCR was carried out with 5′ GG*AGATCT*ATAATGTAC ACTCGTTAC 3′ as the forward primer and 5′ GG*AGATCT*TTAATATGCAGGACCTGT 3′ as the reverse primer. The PCR product carried *Bgl*II sites (shown in italics in the primers) at either end, and was cloned into pAcJR1 (Zuidema *et al*., 1990) to give pAc-BsPH (Fig. 1).

The transfer vector pAc-SePH was constructed by using the polyhedrin gene of SeMNPV as template (van Strien *et al*., 1992). The coding sequence was isolated by PCR from plasmid pSeSDN, containing the 3·6 kbp *Nru*I fragment derived from the *Sph*I-D fragment of SeMNPV DNA. PCR was carried out with 5′ GG*AGATCT*ATAATGTATA-CTCGCTAC 3′ as the forward primer and 5′ GG*AGATCT*TTAA-TAGGCGGGTCCGTT 3′ as the reverse primer. The PCR product carried *Bgl*II sites at either end and was cloned into pAcJR1 to give pAc-SePH (Fig. 1).

As a control, the AcMNPV polyhedrin-coding sequence was amplified by PCR from the plasmid pAcMK10 containing the 7·2 kbp *Eco*RI-I fragment of AcMNPV DNA. PCR was carried out with the forward primer 5′ GG*AGATCT*AATATGCCGGATTACTCA 3′ and the reverse primer 5′ GG*AGATCT*TTAATACGCCGGACCAGT 3′. The PCR product carried *Bgl*II sites at either end and was cloned into pAcJR1 to give pAc-AcPH.

Sf21 cells (2×10^6) were transfected with 5 μg transfer vector and 1 μg *Bsu*36I-linearized BacPAK6 (Kitts & Possee, 1993) DNA by using lipofectin (GIBCO BRL). The recombinant viruses AcMNPV-AcPH, AcMNPV-BsPH and AcMNPV-SePH were purified from polyhedron-containing plaques and their DNA was analysed by restriction enzyme digestion and gel electrophoresis. To ensure that the inserted polyhedrin genes did not contain mutations, PCR was performed with recombinant virus DNA as template and the same primers that were used in the construction of the transfer vectors. The PCR products were cloned into pTZ19R and sequenced.

■ **Protein analysis.** Sf21 cells were infected with wild-type AcMNPV (C6) or with one of the recombinant viruses AcMNPV-AcPH, AcMNPV-BsPH and AcMNPV-SePH at an m.o.i. of 10 $TCID_{50}$ per cell and were harvested at 52 h post-infection (p.i.). The proteins were analysed by SDS–PAGE in a 12·5 % polyacrylamide gel according to the method of van Oers *et al*. (1994). Purified polyhedra of wild-type AcMNPV, SeMNPV and BusuNPV were loaded on the same denaturing gel for comparison of the polyhedrin sizes.

■ **Electron microscopy.** Sf21 cells infected with wild-type AcMNPV or with the recombinant viruses AcMNPV-AcPH, AcMNPV-BsPH and AcMNPV-SePH at an m.o.i. of 10 $TCID_{50}$ per cell were harvested at 52 h p.i. and processed for electron microscopy as described previously (van Lent *et al*., 1990).

■ **Insect bioassays.** Polyhedra from wild-type AcMNPV (Ayres *et al*., 1994) and recombinants were fed to late third-instar *S. exigua* larvae to obtain passage 1 polyhedra for the bioassay. Polyhedra isolated from the infected insects were then used in a bioassay by using the modified droplet-feeding method, as previously described by Hughes & Wood (1981). Suspensions of 10^4, 10^5, 3×10^5, 10^6, 10^7 and 10^8 polyhedra per ml were chosen for wild-type AcMNPV and AcMNPV-AcPH, and suspensions of 10^4, 10^5, 10^6, 10^7, 10^8 and 3×10^8 polyhedra per ml were chosen for AcMNPV-BsPH and AcMNPV-SePH. For each concentration, 36 early third-instar *S. exigua* larvae were tested. Larvae were incubated at 28 °C and mortality was recorded 7 days later. Median doses of ingested polyhedra were calculated from the median volume ingested by a third-instar *S. exigua* larva (0·55 μl) (F. J. J. A. Bianchi, unpublished results) and the polyhedron concentrations of the suspensions. The dose–mortality data were analysed by probit analysis, using the computer program POLO (Russell *et al*., 1977).

Results

Generation of recombinants

To generate AcMNPV recombinants carrying the polyhedrin gene from either BusuNPV or SeMNPV, the transfer vector plasmids pAc-BsPH and pAc-SePH (Fig. 1), containing the polyhedrin-coding sequences from BusuNPV and SeMNPV, respectively, were constructed. Sequence analyses

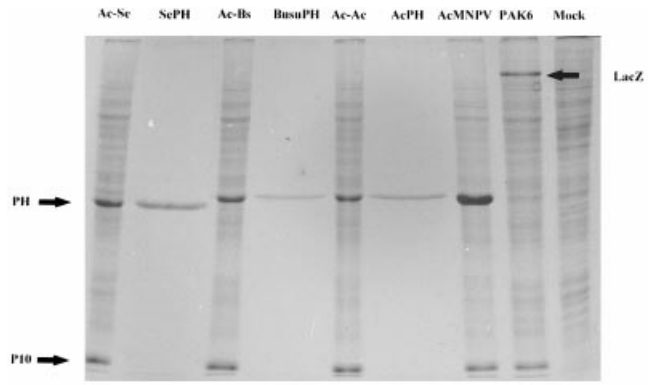

Fig. 2. SDS–PAGE of protein from uninfected Sf21 cells (Mock), Sf21 cells (harvested at 52 h p.i.) infected with parental virus BacPAK6 (PAK6), wild-type AcMNPV-C6 (AcMNPV), AcMNPV-AcPH (Ac-Ac), AcMNPV-BsPH (Ac-Bs) and AcMNPV-SePH (Ac-Se), and polyhedra of wild-type AcMNPV (AcPH), BusuNPV (BusuPH) and SeMNPV (SePH). Protein equivalent to 5×10^4 cells or 1×10^5 polyhedra was loaded per lane. The gel was stained with Coomassie brilliant blue.

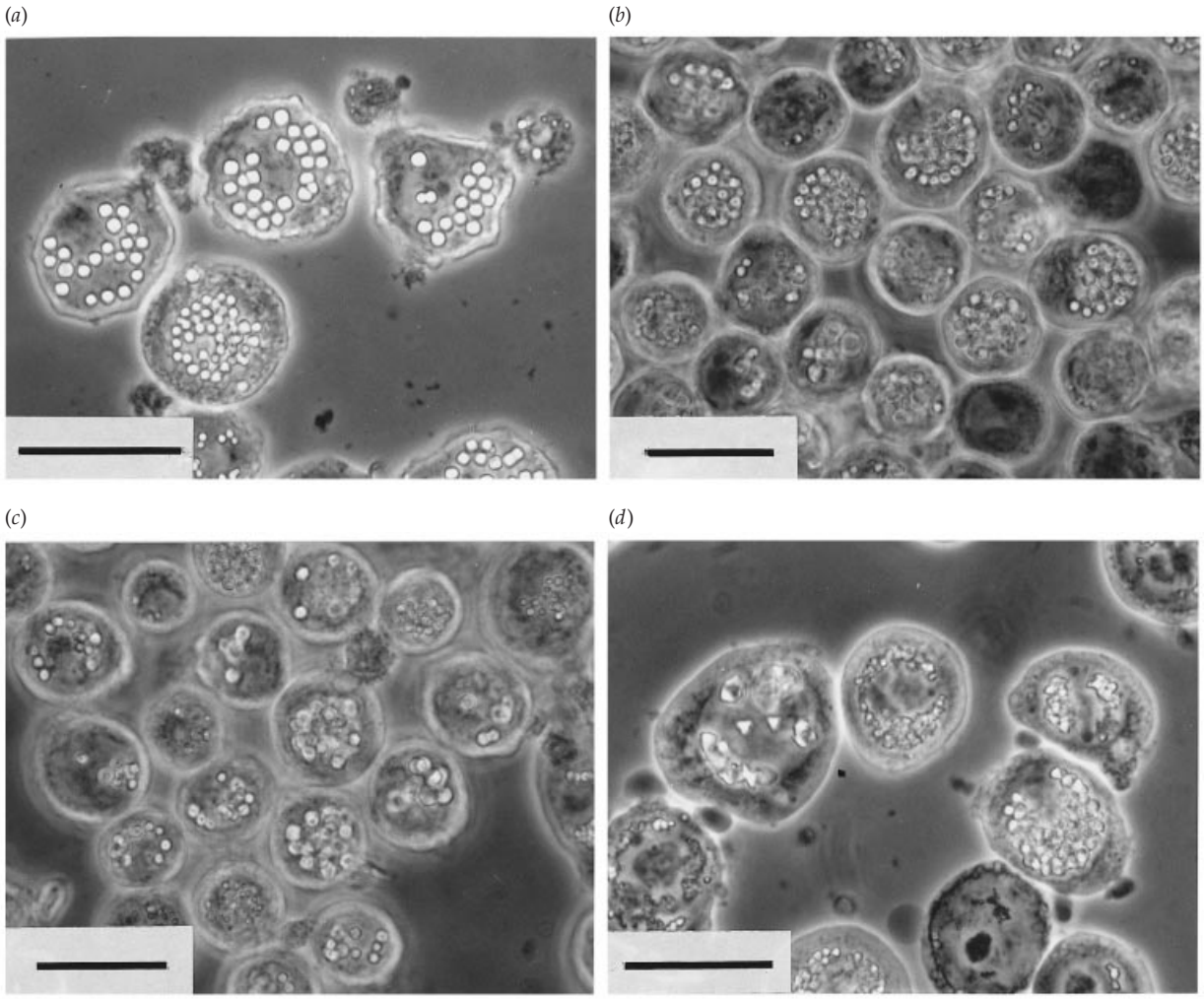

Fig. 3. Phase-contrast microscopy of Sf21 cells infected with wild-type AcMNPV (*a*), AcMNPV-AcPH (*b*), AcMNPV-BsPH (*c*) and AcMNPV-SePH (*d*), at 52 h p.i. Bars represent 20 μm.

confirmed that the polyhedrin genes were inserted correctly into the various transfer vectors. Sf21 cells were co-transfected with either of the vectors and BacPAK6 (Kitts & Possee, 1993) by lipofection. BacPAK6 lacks the polyhedrin gene and recombinants can thus be recognized easily by the appearance of polyhedra. Recombinant AcMNPV-AcPH was made by reintroducing the native polyhedrin-coding sequence into BacPAK6, and was used as a control to assess any cloning artefacts. The recombinants were plaque-purified and their DNA was analysed by restriction enzyme digestion and agarose electrophoresis. The data (not shown) confirmed that the polyhedrin-coding sequences were inserted at the correct location and orientation in BacPAK6. The polyhedrin genes were recloned as PCR products from the respective recombinant viruses and the sequences were found to be identical to the published sequences (Hooft van Iddekinge *et al.*, 1983; van Strien *et al.*, 1992; Hu *et al.*, 1993).

Sf21 cells infected with AcMNPV-AcPH, AcMNPV-BsPH and AcMNPV-SePH were harvested at 52 h p.i. Protein from the cells was analysed by SDS–PAGE and compared with polyhedrin protein from wild-type AcMNPV, BusuNPV and SeMNPV (Fig. 2). The data showed that the polyhedrin genes of BusuNPV, SeMNPV and AcMNPV were correctly expressed by the recombinants and yielded a product of the same size as the polyhedrin present in wild-type BusuNPV, SeMNPV and AcMNPV polyhedra. For example, the size of wild-type SeMNPV polyhedrin is about 2 kDa smaller than that of wild-type AcMNPV (Caballero *et al.*, 1992); this is also the case for the proteins expressed by the recombinants (Fig. 2). The level of polyhedrin expression in all recombinants was high, although slightly lower than that in wild-type AcMNPV-infected cells.

Phase-contrast and electron microscopy

When Sf21 cells were infected with different AcMNPV recombinants, polyhedra could easily be seen in the nuclei by phase-contrast microscopy (Fig. 3). No significant difference in the size and shape of polyhedra could be observed in cells

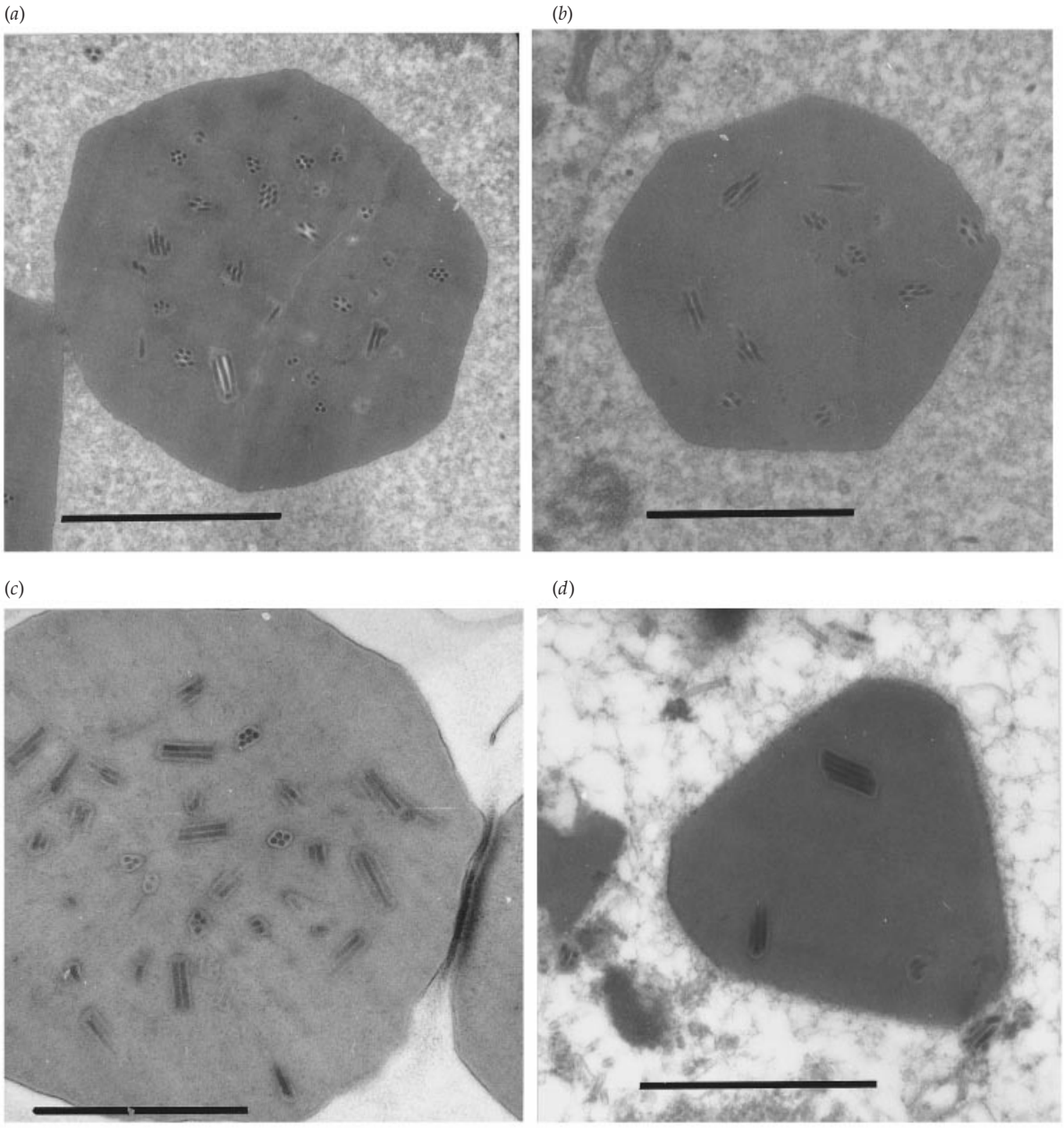

Fig. 4. Electron micrographs of polyhedra representing wild-type AcMNPV (*a*), AcMNPV-AcPH (*b*), AcMNPV-BsPH (*c*) and AcMNPV-SePH (*d*). Bars represent 1 μm.

infected with the AcMNPV wild-type control (Fig. 3*a*), AcMNPV-AcPH recombinant control (Fig. 3*b*) and AcMNPV-BsPH (Fig. 3*c*). The AcMNPV-SePH polyhedra (Fig. 3*d*), however, had a triangular shape, distinct from the wild-type AcMNPV or SeMNPV polyhedra. Polyhedra were released from the infected cells at the end of the infection (data not shown), indicating that the expression of a foreign polyhedrin did not affect nuclear disintegration.

Polyhedra produced by wild-type AcMNPV (Fig. 4 *a*) and by AcMNPV-AcPH (Fig. 4*b*) and AcMNPV-BsPH (Fig. 4*c*) were of normal shape, size and structure, as seen by electron microscopy. Numerous multiple-nucleocapsid virions were observed in sections of these polyhedra and the polyhedra were completed with a calyx. AcMNPV-BsPH did not select for single-nucleocapsid virions (Fig. 4*c*). The polyhedra produced by AcMNPV-SePH had distinct (often triangular) shapes in thin sections and appeared to contain only a few multiple-nucleocapsid virions (Fig. 4*d*). Scanning electron microscopy confirmed the pyramidal shape of these polyhedra. These polyhedra also appeared to be completed with a calyx. Apart from occlusion, other pathogenic features or processes associated with baculovirus infection, e.g. formation of fibrillar

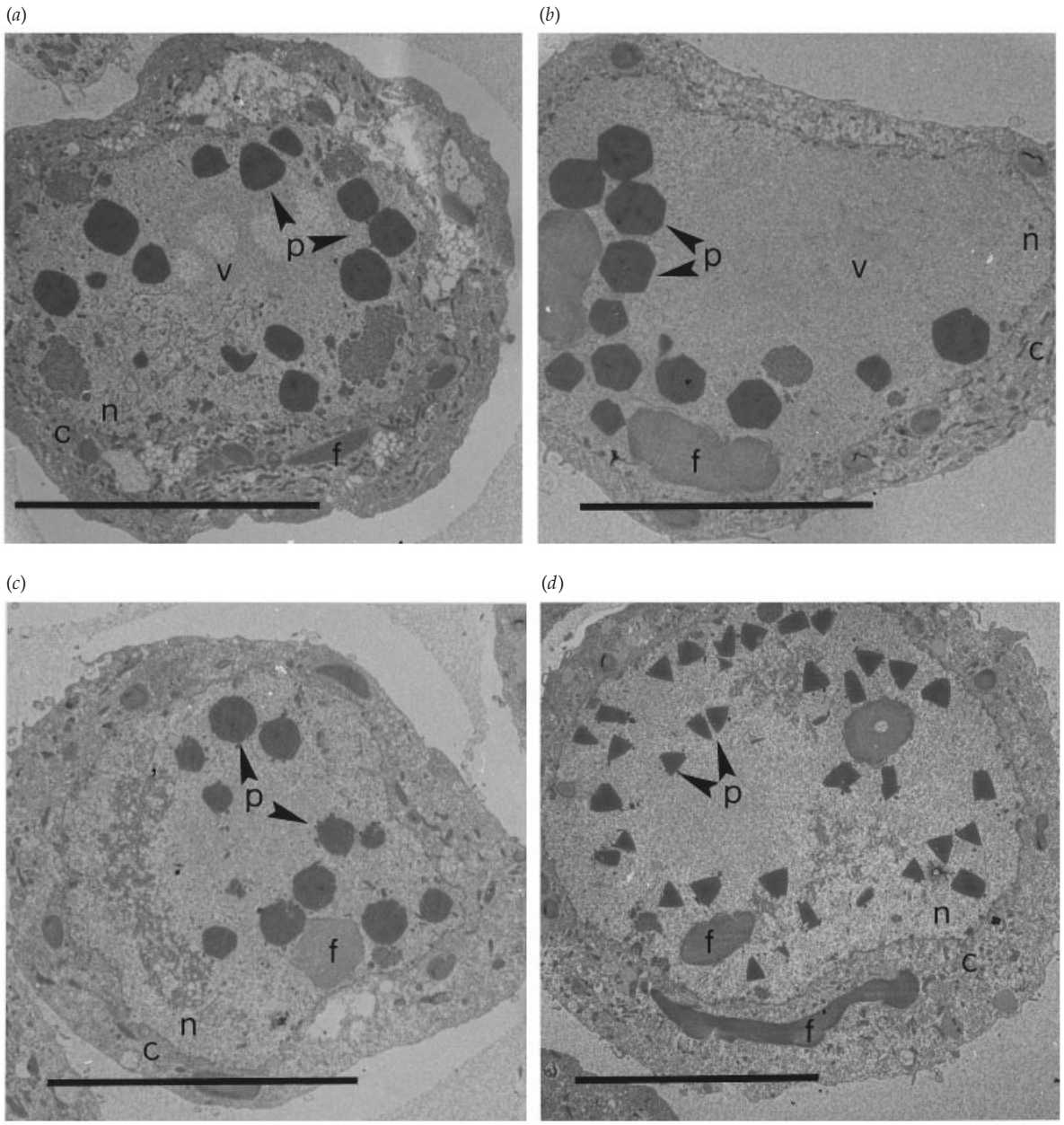

Fig. 5. Electron micrographs of thin sections of Sf21 cells infected with wild-type AcMNPV (*a*), AcMNPV-AcPH (*b*), AcMNPV-BsPH (*c*) and AcMNPV-SePH (*d*). f, Fibrillin; p, polyhedron; n, nucleus; c, cytoplasm; and v, virogenic stroma. Bars represent 10 μm.

structures in the nucleus and cytoplasm, occurrence of virogenic stroma in the nucleus and enlarged nuclei, appeared to be indistinguishable, irrespective of whether recombinants or wild-type AcMNPV were used (Fig. 5).

Physical and biological activity

The alkali-sensitivity was tested in an *in vitro* assay, following the dissolution of polyhedra over time (Zuidema *et al.*, 1989). No difference in alkali-sensitivity between the wild-type and recombinant AcMNPVs was observed (data not shown). Polyhedra from all recombinant NPVs dissolved readily in weak alkali and released ODVs. The remaining polyhedron calyx or 'bag' could be seen from recombinant as well as wild-type AcMNPV.

The infectivity of the various recombinant viruses was determined with a droplet-feeding bioassay by using third-instar *S. exigua* larvae as host, and the results of these assays are shown in Table 1. The various viruses were passaged once in *S. exigua* larvae prior to the assay to eliminate any cell-culture effects. The LD_{50} of AcMNPV-AcPH was not significantly

Table 1. Dose–mortality relationship between wild-type and recombinant AcMNPV in early third-instar *S. exigua* larvae

The dose–mortality data were the result of probit analysis using the POLO computer program (Russell *et al*., 1977). wt, Wild-type; df, degrees of freedom.

Virus	LD_{50} (polyhedra)	90% Confidence limits		Slope	χ^2/df
		Lower	Upper		
AcMNPV-wt	$3{\cdot}5 \times 10^3$	$1{\cdot}4 \times 10^3$	$1{\cdot}2 \times 10^4$	$0{\cdot}933 \pm 0{\cdot}111$	2·25
AcMNPV-AcPH	$5{\cdot}3 \times 10^3$	$2{\cdot}1 \times 10^3$	$2{\cdot}0 \times 10^4$	$0{\cdot}785 \pm 0{\cdot}100$	1·04
AcMNPV-BsPH	$1{\cdot}3 \times 10^4$	$4{\cdot}0 \times 10^3$	$4{\cdot}8 \times 10^4$	$1{\cdot}054 \pm 0{\cdot}125$	6·54
AcMNPV-SePH	$1{\cdot}4 \times 10^5$	$4{\cdot}1 \times 10^4$	$3{\cdot}8 \times 10^6$	$0{\cdot}783 \pm 0{\cdot}143$	1·49

different from that of the wild-type virus AcMNPV-C6 (the parental virus of AcMNV-PAK6), indicating that the cloning process had not affected the biological activity of the virus. The LD_{50} of AcMNPV-BsPH was slightly higher than that of wild-type AcMNPV, whereas the LD_{50} of AcMNPV-SePH was significantly higher than that of the other recombinants and wild-type AcMNPV.

Discussion

Our study indicates that heterologous polyhedrin ORFs, either from an SNPV or an MNPV, can be highly expressed in an AcMNPV–Sf21 expression system and that the polyhedrins are also correctly translocated into the nucleus to form polyhedra. These recombinant polyhedra showed the characteristic paracrystalline lattice structure and were correctly surrounded by a polyhedron envelope (calyx). Jarvis *et al*. (1991) showed that the AcMNPV polyhedrin protein contains domains responsible for nuclear localization and supramolecular assembly. It seems that those domains in the polyhedrin proteins of SeMNPV and BusuNPV are also functional in the AcMNPV–Sf21 cell system, a heterologous genetic environment. Similar results were observed by Roosien *et al*. (1986) and Gonzalez *et al*. (1989) when the polyhedrin genes of *Mamestra brassicae* MNPV and *S. frugiperda* MNPV were introduced into an AcMNPV polyhedrin-deletion mutant by heterologous recombination rather than by targeted insertion. Therefore, the nuclear localization and supramolecular assembly signals of the polyhedrin are likely to be recognized in heterologous NPV–host systems. In the two cases studied, envelopment of the heterologous OBs with a calyx appeared to be unimpeded. Our results showed further that the expression of a foreign polyhedrin did not affect nuclear disintegration, which appears to be a process requiring the interaction of P10 with at least one virus-specific factor (van Oers *et al*., 1994).

Our results contrast with those of Zhou *et al*. (1998). In their paper, the granulin gene of *Trichoplusia ni* GV was used to replace the polyhedrin gene of *Bombyx mori* (Bm) NPV. Polyhedron-like OBs, which did not have paracrystalline lattices and had fragments of the polyhedron envelope wrapped inside, were produced by the resulting recombinant BmNPV in both nucleus and cytoplasm. The amino acid conservation between granulin and lepidopteran polyhedrin is much lower than that between two lepidopteran polyhedrin proteins (e.g. 53% amino acid identity for those of BmNPV/TnGV, 84% for SeMNPV/AcMNPV and 89% for BusuNPV/AcMNPV). It is, therefore, conceivable that granulin has already diverged to such an extent from polyhedrin that it has lost its ability to interact with calyces or ODVs of NPVs. The observation of Zhou *et al*. (1998) that granulin forms polyhedron-like OBs in an NPV background may indicate that the supramolecular assembly mechanisms in NPVs and GVs developed from a common system.

The occlusion process does not appear to be dependent on whether the polyhedrin is from an SNPV or an MNPV, since OBs derived from recombinant AcMNPV producing BusuNPV polyhedrin appear to occlude multiple-nucleocapsid AcMNPV virions quite efficiently and with no apparent loss of infectivity. This suggests that a polyhedrin from an SNPV can recognize ODVs or other MNPV proteins involved in NPV occlusion and OB morphogenesis. Our results also showed that a heterologous polyhedrin from an MNPV (SeMNPV) does not necessarily function better than that from an SNPV (BusuNPV) in an AcMNPV–Sf21 environment.

Polyhedra produced by AcMNPV-SePH were pyramidal in shape and differed profoundly from those produced by wild-type AcMNPV and SeMNPV, which are polyhedral in shape and differ in size. Since it has been reported before that alteration of a single amino acid can cause a major morphological change in polyhedra (Carstens *et al*., 1986, 1987, 1992), several control experiments were carried out to ensure that, in our case, the correct SeMNPV polyhedrin gene was inserted in the recombinant AcMNPV-SePH. First, the inserted polyhedrin sequence in transfer vector pAc-SePH was sequenced and found to be correct, proving that no errors were introduced by the PCR amplification. Second, the transfection and plaque-purification procedures used to generate

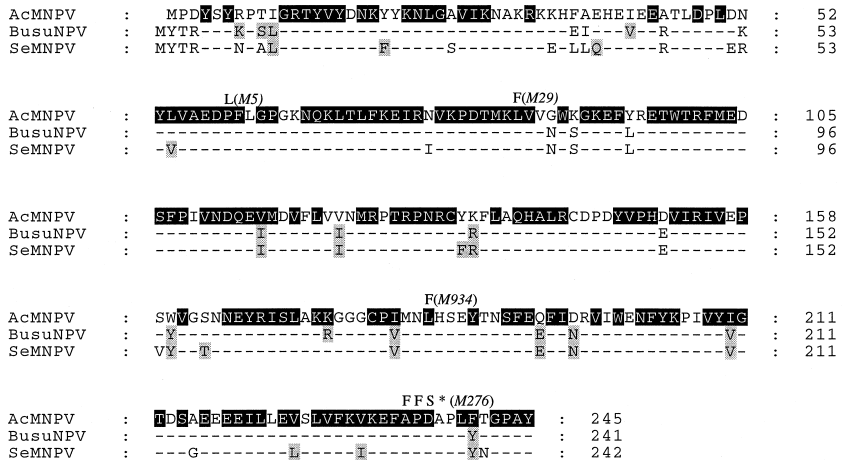

Fig. 6. Alignment of the amino acid sequences of the polyhedrin genes from AcMNPV, BusuNPV and SeMNPV. Black shading in the AcMNPV sequence indicates the conserved amino acid sequence among polyhedrins identified to date (data derived from nucleotide sequences of polyhedrin available in GenBank). The similarity of the BusuNPV and SeMNPV sequences to that of AcMNPV is shown: dashes indicate amino acid identity and grey shading indicates similarity. The locations of four mutations in AcMNPV, M5 (Carstens *et al.*, 1986), M29 (Carstens *et al.*, 1987), M934 and M276 (Carstens *et al.*, 1992), are also indicated.

AcMNPV-SePH were repeated and several plaques were picked and purified separately. In all cases the AcMNPV-SePH polyhedra showed the same, altered morphology. Finally, the polyhedrin gene was amplified from AcMNPV-SePH by PCR and sequenced. The sequence data confirmed that the polyhedrin gene of the AcMNPV-SePH recombinant was identical to that of wild-type SeMNPV (van Strien *et al.*, 1992). Therefore, the explanation for the altered polyhedron morphology of the recombinant AcMNPV-SePH must be found in the expression of the SeMNPV polyhedrin in an AcMNPV–Sf21 environment. When AcMNPV-SePH was amplified in an *S. exigua* cell line (Se-IZD2109) or in *S. exigua* larvae (after injection), the resulting polyhedra still showed an aberrant morphology, suggesting the involvement of a virus-specific factor in the morphogenesis of polyhedra. When *S. exigua* cells were co-infected with AcMNPV-SePH and wild-type SeMNPV at a ratio of 50:1 (final m.o.i. of 10), most of the resulting polyhedra showed the size and shape of wild-type SeMNPV (J. Hajós, unpublished results), suggesting the existence of such a factor.

The infectivity of AcMNPV-BsPH was similar to that of wild-type AcMNPV, indicating that BusuNPV polyhedrin can functionally replace AcMNPV and that the differences in amino acid sequence between AcMNPV and BusuNPV (Fig. 6) have a neutral effect. The infectivity of AcMNPV-SePH for *S. exigua* was low in comparison to wild-type AcMNPV, as reflected by a higher LD_{50}. This may be explained by the fact that fewer virions were occluded in the recombinant polyhedrons containing SeMNPV polyhedrin (Fig. 4*d*). The less efficient occlusion of AcMNPV virions by SeMNPV polyhedrin in comparison to BusuNPV polyhedrin suggests that virion occlusion is a complex and finely controlled process that may be to some extent specific to the polyhedrin protein involved. Indeed, amino acid sequence comparison shows that the polyhedrin of BusuNPV is more closely related to that of AcMNPV than to that of SeMNPV (Fig. 6). Recent experiments expressing the more distantly related granulin gene in BmNPV (Zhou *et al.*, 1998), showing the formation of improperly occluded OBs, support this view.

Although about 30 polyhedrin genes have already been sequenced, little is known about the structure–function relationship of the protein; e.g. its role in crystalline matrix formation and how it interacts with the ODV and with the calyx. Amino acid residues that are conserved in all baculovirus polyhedrin proteins are likely to be functionally important. Often, a single amino acid mutation in polyhedrin can alter the morphology of polyhedra (Carstens *et al.*, 1986, 1987, 1992). All these distinct morphologies were caused by mutations in the conserved regions of the polyhedrin protein (Fig. 6). Our results, especially those obtained with AcMNPV-SePH, suggest that amino acid residues which are not conserved among baculoviral polyhedrin proteins, as well as other virus factors, may play a role in the morphogenesis of OBs and in the efficiency of virion occlusion.

We are grateful to Joop Groenewegen for expert technical assistance with electron microscopy and Magda Usmany for her help with tissue culture. We thank Ine Derksen-Koppers and Magda Usmany for rearing insects and insect cells, respectively. We thank Rob Goldbach and Basil Arif for their continuing interest and critical review of the manuscript. Z. H. Hu is a recipient of a sandwich PhD grant from the Wageningen Agricultural University.

References

Ayres, M. D., Howard, S. C., Kuzio, J., Lopez-Ferber, M. & Possee, R. D. (1994). The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* **202**, 586–605.

Caballero, P., Zuidema, D., Santiago-Alvarez, C. & Vlak, J. M. (1992). Biochemical and biological characterization of four isolates of *Spodoptera exigua* nuclear polyhedrosis virus. *Biocontrol Science and Technology* **2**, 145–147.

Carstens, E. B., Krebs, A. & Gallerneault, C. E. (1986). Identification of an amino acid essential to the normal assembly of *Autographa californica* nuclear polyhedrosis virus polyhedra. *Journal of Virology* **58**, 684–688.

Carstens, E. B., Ye, L.-B. & Faulkner, P. (1987). A point mutation in the polyhedrin gene of a baculovirus, *Autographa californica* MNPV, prevents crystallization of occlusion bodies. *Journal of General Virology* **68**, 901–905.

Carstens, E. B., Williams, G. V., Faulkner, P. & Partington, S. (1992). Analysis of polyhedra morphology mutants of *Autographa californica* nuclear polyhedrosis virus: molecular and ultrastructural features. *Journal of General Virology* **73**, 1471–1479.

Gombart, A. F., Pearson, M. N., Rohrmann, G. F. & Beaudreau, G. S. (1989). A baculovirus polyhedral envelope-associated protein: genetic location, nucleotide sequence, and immunocytochemical characterization. *Virology* **169**, 182–193.

Gonzalez, M. A., Smith, G. E. & Summers, M. D. (1989). Insertion of the SfMNPV polyhedrin gene into an AcMNPV polyhedrin deletion mutant during viral infection. *Virology* **170**, 160–175.

Gross, C. H., Russell, R. L. Q. & Rohrmann, G. F. (1994). *Orgyia pseudotsugata* baculovirus p10 and polyhedron envelope protein genes: analysis of their relative expression levels and role in polyhedron structure. *Journal of General Virology* **75**, 1115–1123.

Harrison, R. L. & Summers, M. D. (1995*a*). Biosynthesis and localization of the *Autographa californica* nuclear polyhedrosis virus 25K gene product. *Virology* **208**, 279–288.

Harrison, R. L. & Summers, M. D. (1995*b*). Mutations in the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus 25 kDa protein gene result in reduced virion occlusion, altered intranuclear envelopment and enhanced virus production. *Journal of General Virology* **76**, 1451–1459.

Hink, F. (1970). Established insect cell line from the cabbage looper, *Trichoplusia ni*. *Nature* **226**, 446–447.

Hooft van Iddekinge, B. J. L., Smith, G. E. & Summers, M. D. (1983). Nucleotide sequence of the polyhedrin gene of *Autographa californica* nuclear polyhedrosis virus. *Virology* **131**, 561–565.

Hu, Z. H., Liu, M. F., Jin, F., Wang, Z. X., Liu, X. Y., Li, M. J., Liang, B. F. & Xie, T. E. (1993). Nucleotide sequence of the *Buzura suppressaria* single nucleocapsid nuclear polyhedrosis virus polyhedrin gene. *Journal of General Virology* **74**, 1617–1620.

Hu, Z. H., Arif, B. M., Jin, F., Martens, J. W. M., Chen, X. W., Sun, J. S., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1998). Distinct gene arrangement in the *Buzura suppressaria* single-nucleocapsid nucleopolyhedrovirus genome. *Journal of General Virology* **79**, 2841–2851.

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

Jarvis, D. L., Bohlmeyer, D. A. & Garcia, A., Jr (1991). Requirements for nuclear localization and supramolecular assembly of a baculovirus polyhedrin protein. *Virology* **185**, 795–810.

Jarvis, D. L., Bohlmeyer, D. A. & Garcia, A., Jr (1992). Enhancement of polyhedrin nuclear localization during baculovirus infection. *Journal of Virology* **66**, 6903–6911.

Kitts, P. A. & Possee, R. D. (1993). A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques* **14**, 810–817.

Minion, F. C., Coons, L. B. & Broome, J. R. (1979). Characterization of the polyhedral envelope of the nuclear polyhedrosis virus of *Heliothis virescens*. *Journal of Invertebrate Pathology* **34**, 303–307.

Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A. & Summers, M. D. (editors) (1995). *Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses*. Vienna & New York: Springer-Verlag.

Rohrmann, G. F. (1986). Polyhedrin structure. *Journal of General Virology* **67**, 1499–1513.

Rohrmann, G. F. (1992). Baculovirus structural proteins. *Journal of General Virology* **73**, 749–761.

Roosien, J., Usmany, M., Klinge-Roode, E. C., Meijerink, P. H. S. & Vlak, J. M. (1986). Heterologous recombination between baculoviruses. In *Fundamental and Applied Aspects of Invertebrate Pathology*, pp. 389–392. Edited by R. A. Samson, J. M. Vlak & D. Peters. Wageningen: Foundation of the Fourth International Colloquium of Invertebrate Pathology.

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.

van Lent, J. W. M., Groenen, J. T. M., Klinge-Roode, E. C., Rohrmann, G. F., Zuidema, D. & Vlak, J. M. (1990). Localization of the 34 kDa polyhedron envelope protein in *Spodoptera frugiperda* cells infected with *Autographa californica* nuclear polyhedrosis virus. *Archives of Virology* **111**, 103–114.

van Oers, M. M., Flipsen, J. T. M., Reusken, C. B. E. M. & Vlak, J. M. (1994). Specificity of baculovirus p10 functions. *Virology* **200**, 513–523.

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.

Vaughn, J. L., Goodwin, R. H., Tompkins, G. J. & McCawley, P. (1977). The establishment of two cell lines from the insect *Spodoptera frugiperda* (*Lepidoptera*; *Noctuidae*). *In Vitro* **13**, 213–217.

Vlak, J. M. & Rohrmann, G. F. (1985). The nature of polyhedrin. In *Viral Insecticides for Biological Control*, pp. 489–544. Edited by K. Maramorosh & K. E. Sherman. Orlando: Academic Press.

Whitt, M. A. & Manning, J. S. (1988). A phosphorylated 34-kDa protein and a subpopulation of polyhedrin are thiol linked to the carbohydrate layer surrounding a baculovirus occlusion body. *Virology* **163**, 33–42.

Zhou, C. E., Ko, R. & Maeda, S. (1998). Polyhedron-like inclusion body formation by a mutant nucleopolyhedrovirus expressing the granulin gene from a granulovirus. *Virology* **240**, 282–294.

Zuidema, D., Klinge-Roode, E. C., van Lent, J. W. M. & Vlak, J. M. (1989). Construction and analysis of an *Autographa californica* nuclear polyhedrosis virus mutant lacking the polyhedral envelope. *Virology* **173**, 98–108.

Zuidema, D., Schouten, A., Usmany, M., Maule, A. J., Belsham, G. J., Roosien, J., Klinge-Roode, E. C., van Lent, J. W. M. & Vlak, J. M. (1990). Expression of cauliflower mosaic virus gene I in insect cells using a novel polyhedrin-based baculovirus expression vector. *Journal of General Virology* **71**, 2201–2209.

Received 27 August 1998; Accepted 18 December 1998