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The single-nucleocapsid nucleopolyhedrovirus of *Buzura suppressaria* encodes a P10 protein

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The *p10* gene of *Buzura suppressaria* single-nucleocapsid nucleopolyhedrovirus (BusuNPV) was identified by virtue of its localization downstream from the *Autographa californica* (Ac) MNPV *p26* homologue. The BusuNPV *p10* gene encodes a protein of 94 amino acids. The amino acid sequence contains domains characteristic of baculovirus P10 proteins, e.g. a coiled-coil domain, a proline-rich motif and a positively charged C terminus. The highest amino acid homologies were found with the *Spodoptera littoralis* (Spli) NPV and *Spodoptera exigua* (Se) MNPV P10 proteins. An AcMNPV recombinant expressing the BusuNPV P10 formed fibrillar structures in the cytoplasm of *Spodoptera frugiperda* cells. BusuNPV P10 could not fully replace AcMNPV P10 in its nuclear disintegration function, since polyhedra were not efficiently liberated from infected cells late in infection. The BusuNPV *p26* gene encodes a protein of 263 amino acid residues with 70% amino acid similarity with SeMNPV P26. Downstream of the BusuNPV *p10* gene, the gene for the occlusion-derived virus protein ODVP-6e is located. This is unlike the situation in many other NPVs, including SeMNPV, where the *p10* gene neighbours the *p74* gene. The data presented here suggest that although the *p10* gene is not conserved in sequence, evolutionary pressure preserves the structure of P10 and hence its function. These data also indicate that all NPVs, MNPVs as well as SNPVs, contain this gene.

Introduction

Polyhedrin and P10 are the major late proteins in insect cells infected with multiple-nucleocapsid nucleopolyhedroviruses (MNPVs). P10 is not a structural component of the virus, but is found as fibrillar structures in the cytoplasm and nucleus of infected insect cells (Croizier *et al.*, 1987; Vlak *et al.*, 1988; Williams *et al.*, 1989; van Oers *et al.*, 1994). It is involved in the release of polyhedra from infected cell-nuclei late in infection (van Oers *et al.*, 1993) and plays a role in polyhedron morphogenesis (Gross *et al.*, 1994; Lee *et al.*, 1996). Results obtained from both sequence and mutational analyses have led to a general model for structural and functional domains in P10 for which the name fibrillin was proposed (van Oers & Vlak, 1997). For the first domain, which comprises approximately the N-terminal half of P10, a coiled-coil structure was predicted (van Oers, 1994; Wilson *et al.*, 1995). This region is probably involved in intermolecular interactions leading to aggregation of P10 molecules. A second domain consists of a short proline-rich sequence that may be responsible for the liberation of polyhedra from the nuclei of infected cells. At the C terminus, a positively charged domain is present that is necessary for the alignment of P10 aggregates into fibrillar structures (van Oers *et al.*, 1993).

So far, only *p10* sequences from MNPVs have been reported and the predicted P10 proteins show a high degree of variability in amino acid sequence (Kuzio *et al.*, 1984; Leisy *et al.*, 1986; Chou *et al.*, 1992; Zuidema *et al.*, 1993, Hu *et al.*, 1994; Wilson *et al.*, 1995; Zhang *et al.*, 1995; Faktor *et al.*, 1997). The variability among *p10* genes complicates their

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identification in other baculoviruses. In this paper a *p10* homologue is described in the single-nucleocapsid nucleopolyhedrovirus (SNPV) of *Buzura suppressaria* (BusuNPV). *B. suppressaria* is a major pest insect in tea in China (Xie *et al.*, 1979) and a molecular analysis of its SNPV has been initiated. The sequence of the polyhedrin gene of this virus has been published by Hu *et al.* (1993).

A gene encoding a protein of 26 kDa (P26) is located upstream of the *p10* gene in several MNPV genomes (Bicknell *et al.*, 1987; Zuidema *et al.*, 1993; Poloumienko & Krell, 1997; van Strien *et al.*, 1997). This conserved gene arrangement was used to locate a putative *p10* gene in BusuNPV. Once identified, the authenticity and functionality of this SNPV *p10* homologue were tested by expressing it from the AcMNPV genome. (See Fig. 3 legend for baculovirus abbreviations.)

Methods

■ **Sequence analysis.** The *Buzura suppressaria* NPV (BusuNPV) isolate, also known as BsSNPV, was described by Xie *et al.* (1979). A DNA library was made by cloning *Hin*dIII, *Eco*RI and *Bam*HI restriction fragments of the BusuNPV genome into pTZ19R. Random sequence analysis was performed with standard forward and reverse sequencing primers. The resulting sequences were compared with known baculoviral sequences by using UWGCG FASTA programs (versions 8.1 and 9.0). Based on these results, the sequence of the cloned *Eco*RI-E (pHZH50) and *Hin*dIII-D (pSH25) fragments (Fig. 1) was further analysed with specific primers to obtain the complete sequence of the putative *p26* and *p10* genes. The region upstream of *p26* was sequenced up to the *Bam*HI site in the *Hin*dIII-D fragment.

■ **Transfer of the BusuNPV *p10* coding sequence to the AcMNPV genome.** The BusuNPV *p10* coding sequence was obtained by PCR technology. The cloned *Eco*RI-E fragment (pHZH50) was used as template and the oligonucleotides 5′ CGGGATCCATCATGTCGCAAAATATTTG 3′ and 5′ CGGGATCCTTATTTTTTCAATCCAGTG 3′ as up- and downstream primers, respectively. In this way, the BusuNPV *p10* sequence was amplified from nucleotide residue −3 to +285, relative to the ATG translational start site, and *Bam*HI restriction sites were introduced at both ends of this sequence (see Fig. 4*a*). The resulting PCR product was digested with *Bam*HI and cloned into pTZ18R. The integrity of the cloned PCR product was confirmed by sequence analysis. Subsequently, it was recloned as a *Bam*HI fragment into plasmid pAcAS3 (Vlak *et al.*, 1990) downstream of the AcMNPV *p10* promoter to generate transfer vector pAcMVO7. This transfer vector contains a gene cassette consisting of the *Drosophila melanogaster* hsp70 promoter, the *E. coli lacZ* coding sequence and an SV40 transcriptional terminator, to enable the selection of recombinant viruses. The orientation of the insert was verified by restriction enzyme digestion with *Sph*I and *Ava*II (Fig. 4*a*) and by sequence analysis.

Plasmid pAcMVO7 was cotransfected into Sf21 cells by lipofectin-mediated transfection with DNA of the *p10*-negative parental virus AcMO21, which had been linearized at the *p10* locus with *Bsu*36I (Fig. 4*b*; Martens *et al.*, 1995). Recombinant viruses were selected based on their β-galactosidase expression, plaque-purified and high-titre stocks were prepared using standard techniques (King & Possee, 1992). The identity of the resulting recombinant virus AcMVO7 was analysed by restriction enzyme analysis and by PCR using the oligonucleotide 5′ GGTCTAGACTGTGTGCAATTGCCGTAC 3′, which hybridizes upstream of the AcMNPV *p10* promoter, and the downstream BusuNPV *p10* primer mentioned above.

As a control in the experiments, recombinant AcMO16 was used (Fig. 4*b*; van Oers *et al.*, 1993). This recombinant contains the AcMNPV *p10* sequence from −3 to +282 nt in the same up- and downstream context as the BusuNPV *p10* sequence in the recombinant AcMVO7.

■ **Protein analysis.** *Spodoptera frugiperda* (Sf21) cells (Vaughn *et al.*, 1977) were grown and maintained in Hink's insect medium (Hink, 1970) supplemented with 10% foetal calf serum at 27 °C. Cells were infected at

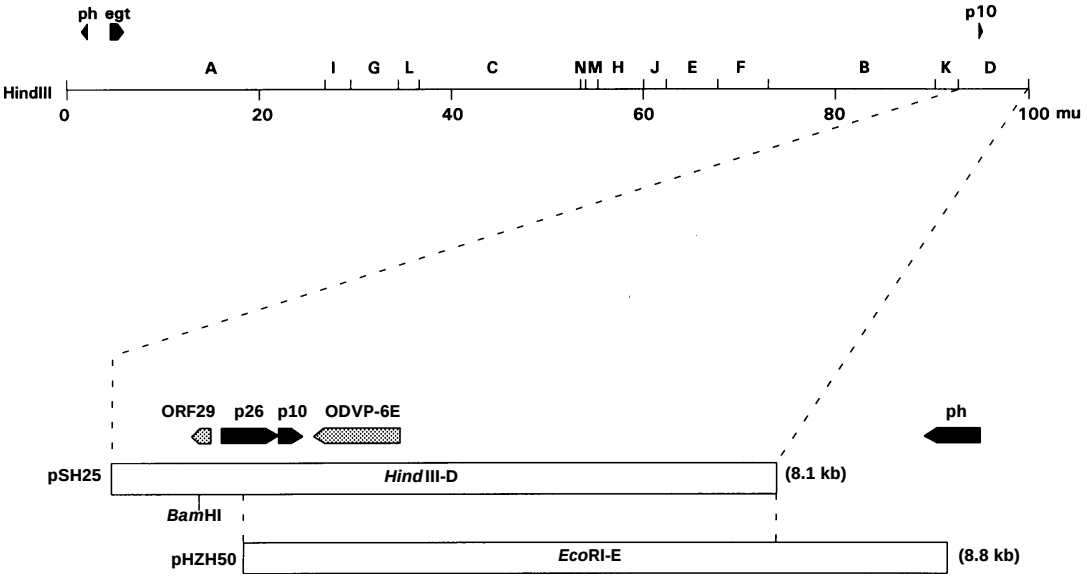

Fig. 1. Linear physical map of the BusuNPV genome for restriction endonuclease *Hin*dIII. Below, the cloned *Hin*dIII-D (pSH18) and *Eco*RI-E (pHZH50) fragments are indicated together with the genes identified thus far on these fragments. ph, polyhedrin.

an m.o.i. of 10 $TCID_{50}$ units with AcMNPV wild-type (wt), the *p10* deletion mutant AcMO21, the recombinant AcMVO7 encoding BusuNPV P10 and the control recombinant AcMO16. Infected cells were harvested at 48 h post-infection (p.i.), washed twice with PBS and resuspended in PBS supplemented with 120 mM Tris–HCl, pH 6·8; 1·25% SDS, 425 mM mercaptoethanol, 6% (w/v) Ficoll and 0·001% (w/v) bromophenol blue. The protein pattern was analysed in a 16·5% Tricine–SDS–PAGE system, according to Schägger & von Jagow (1987), enabling the separation of polypeptides ranging from 10 to 40 kDa.

■ **Phase-contrast and electron microscopy.** Sf21 cells were infected with the recombinants AcMO16, AcMO21 and AcMVO7 at an m.o.i. of 10 $TCID_{50}$ units and incubated at 27 °C. Infected insect-cell cultures were examined at 5 days p.i. with a Leitz Labovert phase-contrast microscope. For electron microscopy, infected cells were harvested at 48 h p.i. and embedded as described by van Lent *et al.* (1990). Ultrathin sections were cut with a Reichert-Jung Ultracut microtome and examined with a Philips CM12 electron microscope.

Results

The BusuNPV *p26* gene

Randomly cloned restriction fragments of BusuNPV DNA were partially sequenced with standard primers hybridizing to vector sequences. In this way, the 3′ sequence of the *p26* gene was found at one end of the 8·8 kbp *Eco*RI-E fragment by comparison with known baculovirus sequences (Fig. 1). Previously, the other end of this fragment was shown to harbour polyhedrin gene sequences (Hu *et al.*, 1993). The sequence of the 3′ end of the *p26* gene was completed with specific primers hybridizing internally in *p26*. The 5′ end of *p26* and its upstream flanking region were obtained by sequencing the *Hin*dIII-D fragment up to the internal *Bam*HI site (Fig. 1).

The BusuNPV *p26* gene has an open reading frame (ORF) of 789 nt, potentially encoding a protein of 263 amino acids with a putative mass of 30·7 kDa (Fig. 2). Upstream of the *p26* ORF, a TAAG motif characteristic for baculovirus late promoters is located at −15 nt relative to the putative ATG start codon. Further upstream, at position −141, a CAGT motif is found, characteristic of baculovirus early gene mRNA start sites (Blissard *et al.*, 1992; Pullen & Friesen, 1995). The amino acid sequence of the predicted P26 protein was aligned with other known P26 sequences (data not shown) and showed greatest identity to SeMNPV P26 (69·7%). Several amino acid sequences are well-conserved in P26, like the HQFPGV, GAPI, LVSVVT, SVYG and QLPY motifs (Fig. 2, printed in bold). The function of P26 in the infection process is not clear yet, but it is non-essential for AcMNPV replication in cell culture (Rodems & Friesen, 1993). Screening the EMBL and GenBank databases for sequences homologous to either the complete P26 or the conserved motifs mentioned above did not provide further information as to the possible function of P26. Upstream of the *p26* gene, a partially sequenced ORF is located in the opposite orientation that extends beyond the *Bam*HI site and shows homology with the AcMNPV ORF 29 (Ayres *et al.*, 1994).

The BusuNPV *p10* gene

In order to find the BusuNPV *p10* gene, the region downstream of the *p26* gene was sequenced. This strategy was based on the collinear arrangement of *p26* and *p10* genes in several MNPVs. An ORF of 282 nt was found downstream of *p26* on the *Eco*RI-E fragment (Fig. 1), encoding a putative protein of 94 amino acids with a predicted molecular mass of 10·2 kDa. It showed greatest identity to the SpliMNPV (63·2% identity) and SeMNPV (61·9% identity) P10 proteins (Fig. 3). The amino-terminal half of the predicted protein contains several heptad-repeat sequences, in which the first and fourth amino acid are occupied by hydrophobic amino acids, as has been found in other P10 proteins (Fig. 3, dark shading). Like P10 proteins of MNPVs, the BusuNPV P10 protein has a proline-rich domain (PEIPDLPDVP; Fig. 3, shaded light-grey), where prolines are surrounded by hydrophobic and negatively charged residues and a positively charged carboxy-terminal domain (RKTGTGLKK; Fig. 3, shaded dark-grey).

The 5′ flanking region of BusuNPV *p10* contains a consensus baculovirus late promoter motif, TAAG, that overlaps with the translational stop codon (TAA) of the *p26* ORF. Thirty-five nucleotides separate the TAAG motif and the ATG start codon. The use of the TAAG motif as transcriptional start site would result in a *p10* transcript with a short, unstructured 5′ untranslated region (UTR) with a GC content of approximately 20%. At the 3′ end, a putative polyadenylation signal (AATAAA) was found encompassing the translational stop codon. An alternative signal (ATTAAA) is present 233 nt downstream of the TAA stop codon. This ATTAAA motif is followed by GT-rich sequences, commonly found 30–40 nt downstream of active poly(A) signals (Edwalds-Gilbert *et al.*, 1997). The two ATTAAA motifs present in the BusuNPV *p10* ORF are not followed by such GT-rich sequences.

Another ORF (Fig. 1), with homology to the AcMNPV 'occlusion derived viral protein' gene (*odvp-6e*) (Theilmann *et al.*, 1996), was found downstream of the *p10* gene (Xinwen Chen, personal communication). This ORF is oriented in the opposite direction to *p10* and is separated from the *p10* coding sequence by 100 nt. This means that BusuNPV differs from AcMNPV, BmNPV, CfMNPV, OpMNPV and SeMNPV in that in the genome the *p10* gene is not followed by the *p74* gene (Kuzio *et al.*, 1989; Leisy *et al.*, 1986; Hill *et al.*, 1993; Zuidema *et al.*, 1993; Palhan & Gopinathan, 1996). The BusuNPV *p74* gene was found in another part of the genome (Hu Zhihong, personal communication).

Functionality of BusuNPV P10

Due to the absence of a system to generate BusuNPV recombinants or deletion mutants, the functionality of the BusuNPV P10 protein was studied in Sf21 cells by replacing the AcMNPV *p10* coding sequence with that of BusuNPV. To

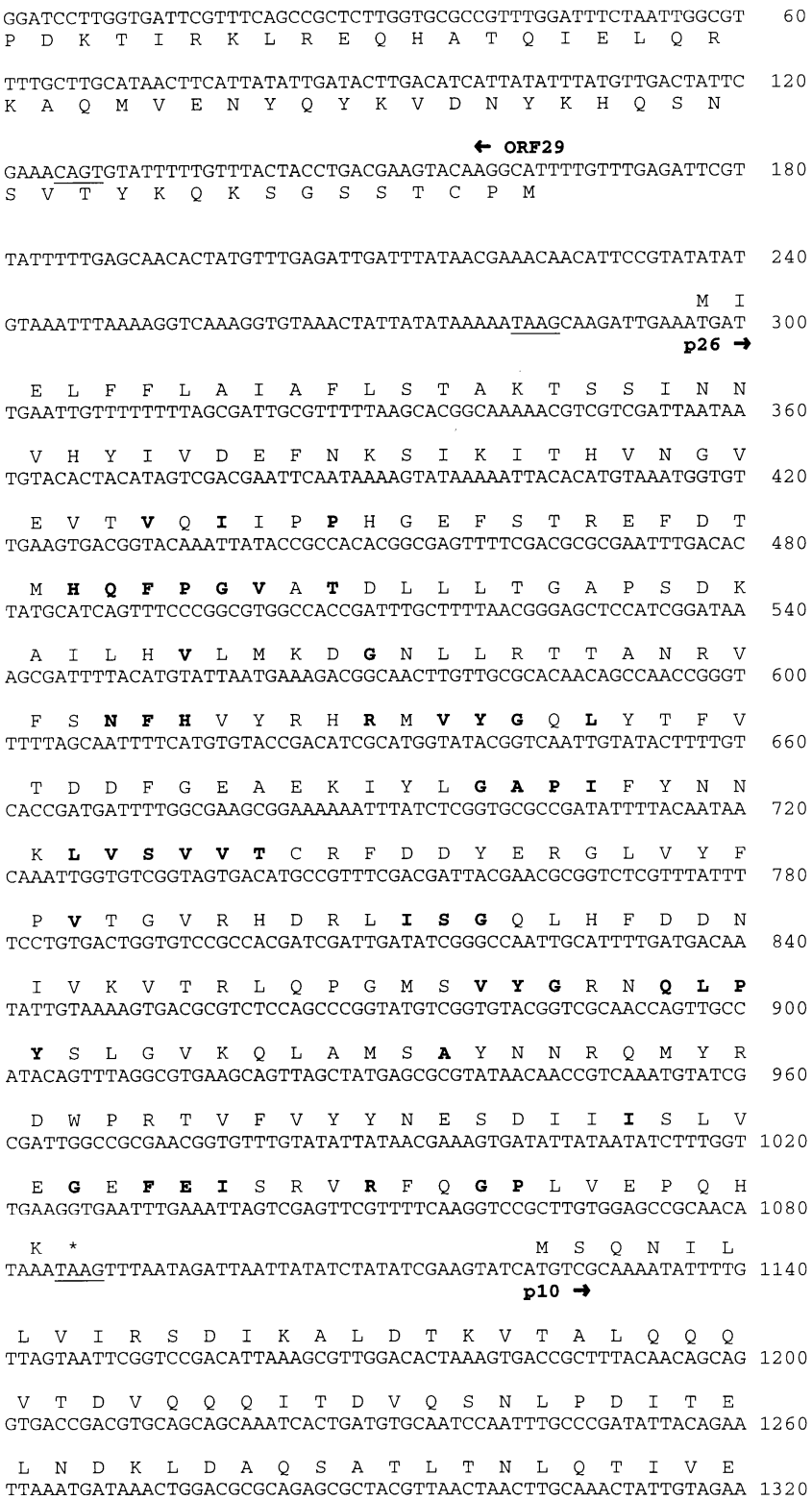

Fig. 2. For legend see facing page.

this end the BusuNPV *p10* coding sequence was obtained by PCR technology and cloned under control of the AcMNPV *p10* promoter in the transfer vector pAcAS3 (Vlak *et al.*, 1990). The recombinant virus AcMVO7 was made (Fig. 4*b*) by recombination with the AcMNPV *p10* deletion mutant AcMO21 (Martens *et al.*, 1995). As a positive control, the

Fig. 2. Nucleotide sequence of a 1680 bp region of the BusuNPV genome, starting from the *Bam*HI site in the *Hin*dIII-D fragment and containing the *p26* and *p10* genes. The *p26* ORF starts at residue 296 and terminates at residue 1085. Conserved amino acid residues in the P26 protein are printed in bold. The *p10* coding sequence starts at residue 1123 and ends at residue 1405. The CAGT, TAAG and putative poly(A) motifs are underlined. The BusuNPV *odvp-6e* homologue is located downstream of *p10* in the opposite orientation.

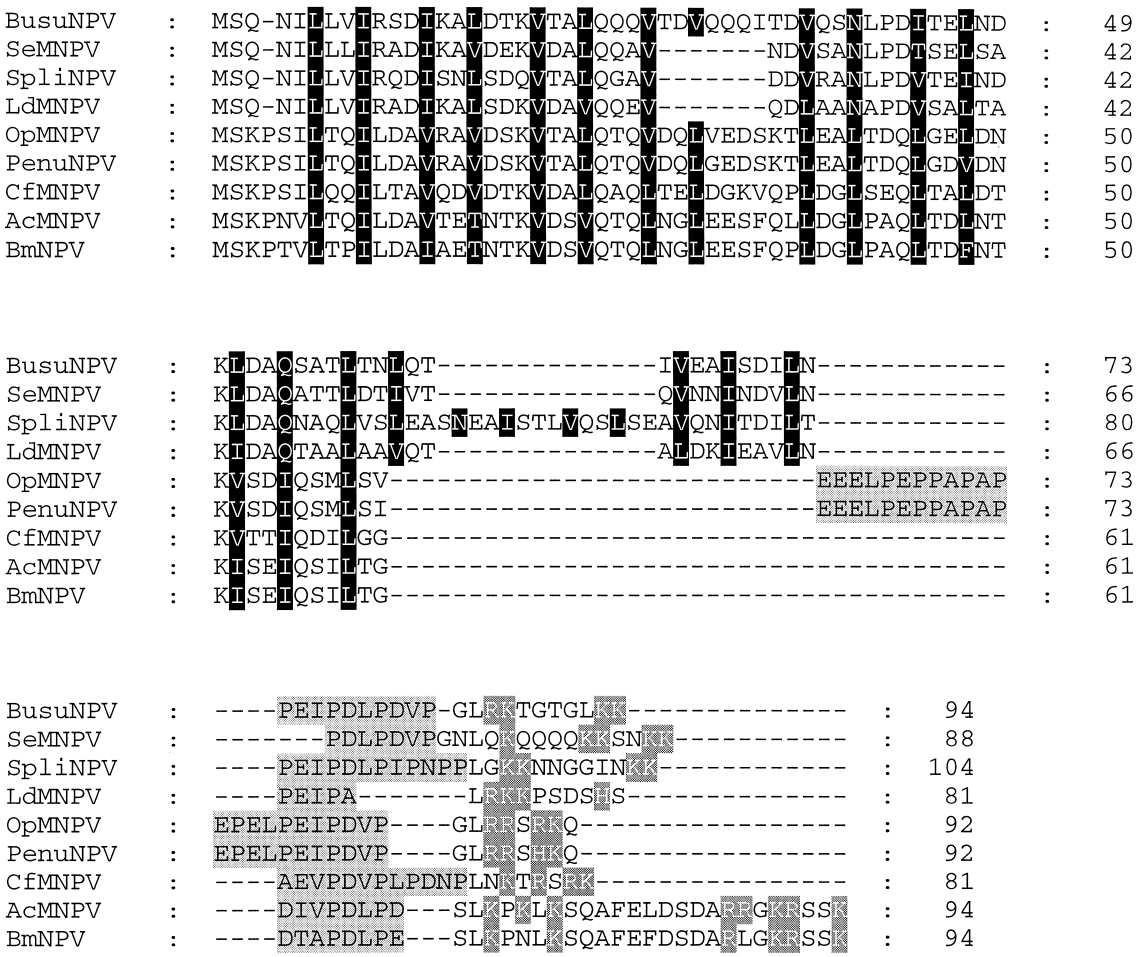

Fig. 3. Sequence alignment of BusuNPV P10 with the P10 proteins of other nucleopolyhedroviruses. Black shading is used to indicate positions 1 and 4 in heptad-repeat sequences in the amino-terminal domain, light-grey shading denotes a conserved proline-rich domain and dark-grey is used to show the positively charged residues in the C-terminal region of the various P10 proteins. Baculovirus abbreviations: BusuNPV, *Buzura suppressaria* NPV; SeMNPV, *Spodoptera exigua* MNPV; SpliNPV, *Spodoptera littoralis* NPV; LdMNPV, *Lymantria dispar* MNPV; OpMNPV, *Orgyia pseudotsugata* MNPV; PenuNPV, *Perina nuda* NPV; CfMNPV, *Choristoneura fumiferana* MNPV; AcMNPV, *Autographa californica* MNPV; BmNPV, *Bombyx mori* NPV.

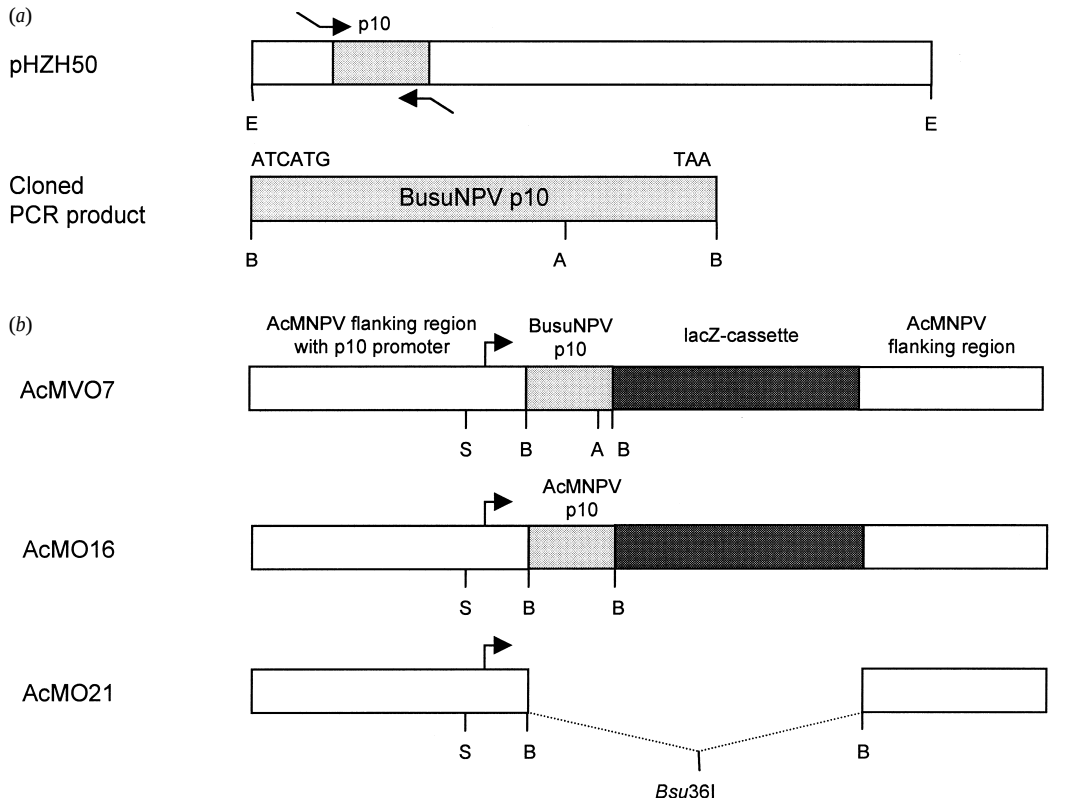

Fig. 4. (*a*) Schematic overview of the amplification of the BusuNPV *p10* coding sequence with the cloned *Eco*RI-E fragment used as template in the PCR. The arrows indicate the positions of the primers. (*b*) Representation of the *p10* locus in the recombinant AcMVO7, encoding BusuNPV P10, the AcMNPV P10-encoding recombinant AcMO16 and the *p10* deletion mutant AcMO21. A, *Ava*II; B, *Bam*HI; E, *Eco*RI; S, *Sph*I. (*c*) 16·5% Tricine–SDS–PAGE of Sf21 cells (1) infected for 48 h with wild-type AcMNPV (2), AcMO16 (3), AcMO21 (4) and AcMVO7 (5). Each lane contains the equivalent of $3{\cdot}5 \times 10^4$ cells. Molecular mass markers are indicated in kDa.

recombinant AcMO16 (van Oers *et al.*, 1993) was used, in which the AcMNPV *p10* ORF is in the same up- and downstream sequence context as the BusuNPV *p10* ORF in AcMVO7. Infection of Sf21 cells with the recombinant AcMVO7 led to synthesis of a protein of approximately 10 kDa (Fig. 4*c*, lane 5) that was not observed in cells infected with the *p10* deletion mutant AcMO21 (lane 4). The BusuNPV P10 protein is comparable in size to AcMNPV P10 (lanes 2 and 3), as was expected from the sequence data (Figs 2 and 3).

Sf21 cells infected with the recombinant AcMVO7 were analysed by electron microscopy to see whether P10-specific structures were formed. Fibrillar structures were observed in the cytoplasm (Fig. 5*a*) and resembled fibrillar structures found in AcMNPV- and AcMO16-infected cells (Fig. 5*d*). In the nucleus of AcMVO7-infected cells, large amorphous structures were formed (Fig. 5*b*). These structures differed significantly from those in the cytoplasm and from the nuclear, fibrillar structures known from AcMNPV P10. The amorphous, nuclear structures were clearly distinct from the virogenic stroma and appeared to interact with electron-dense spacers (Fig. 5*b*) like fibrillar structures in AcMNPV-infected cells. Both fibrillar and amorphous structures must be induced by the BusuNPV P10 homologue, since they were absent from cells infected with the *p10* deletion mutant AcMO21 (data not shown). The polyhedra of the recombinant are surrounded by polyhedral envelopes (Fig. 5*c*) as in wild-type AcMNPV infections.

One of the functions ascribed to P10 is the release of occlusion bodies from the nuclei of infected cells (van Oers *et al.*, 1993). In order to study this function of BusuNPV P10, Sf21 cells were infected with AcMVO7, AcMO16, and with the

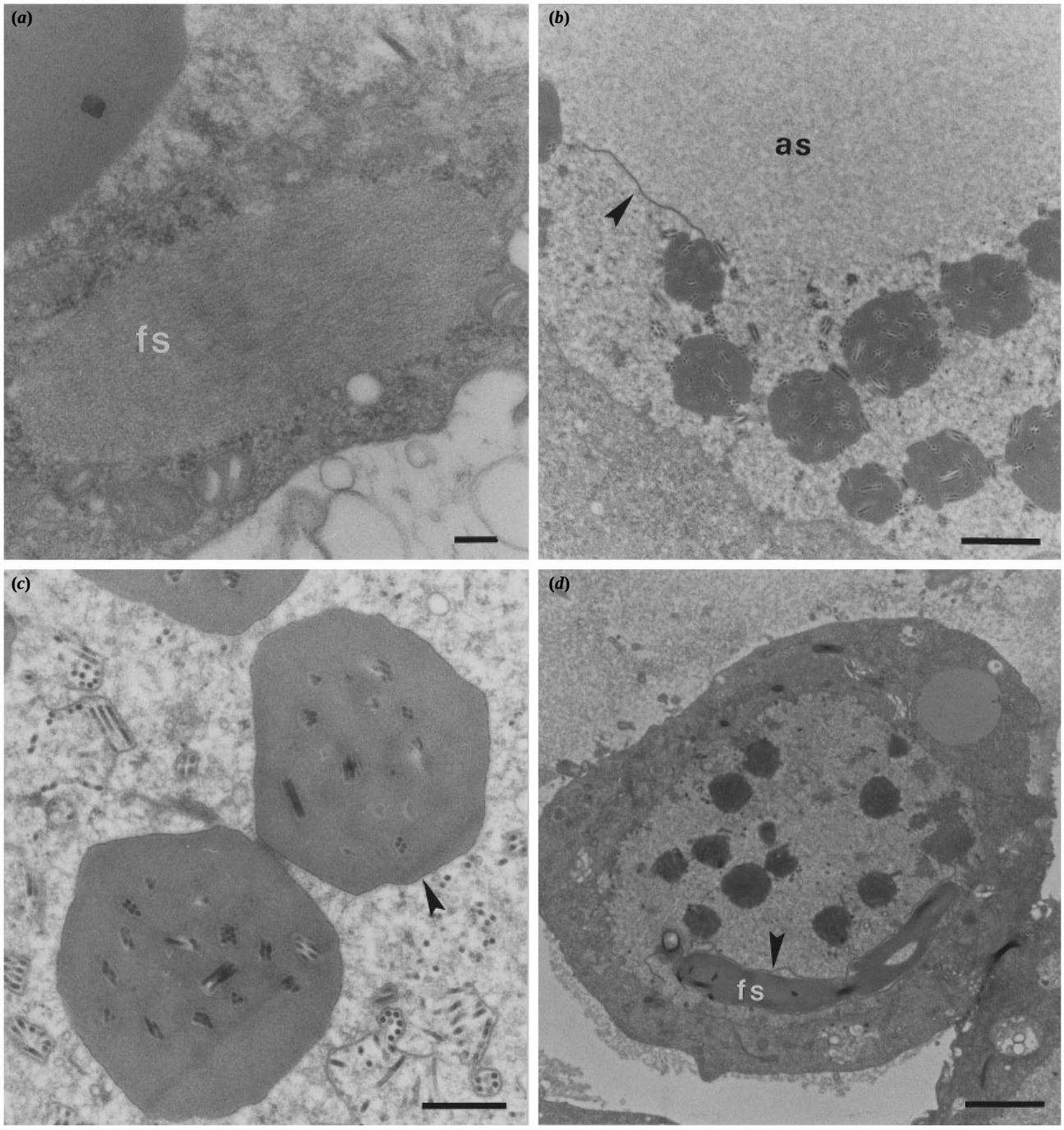

Fig. 5. Electron microscopic images of Sf21 cells 48 h p.i. with AcMVO7, expressing BusuNPV P10, and AcMO16, expressing AcMNPV P10. (*a*) Cytoplasmic fibrillar structure in AcMVO7-infected cells; (*b*) amorphous nuclear structure induced by AcMVO7; (*c*) AcMVO7 polyhedra surrounded by polyhedron envelopes; (*d*) nuclear and cytoplasmic fibrillar structures in Sf21 cells infected with AcMO16. fs, fibrillar structure; as, amorphous structure; arrows indicate electron-dense spacers (*b*, *d*) or the polyhedral envelope (*c*). The bars correspond to 0·2 μm, 1 μm, 0·5 μm and 2 μm in (*a*), (*b*), (*c*) and (*d*), respectively.

p10 deletion mutant AcMO21. Five days p.i. cells were examined for the release of polyhedra (Fig. 6). Cells infected with AcMO16 (Fig. 6*a*) released large numbers of polyhedra due to the presence of wild-type P10 protein. Cells infected with the *p10* deletion mutant AcMO21 (Fig. 6*b*) did not release polyhedra. The recombinant AcMVO7 expressing BusuNPV P10 (Fig. 6*c*) released very few polyhedra from infected cell nuclei, as compared to AcMO16. Even at 10 days p.i., the majority of polyhedra were still captive within the nuclei (data not shown).

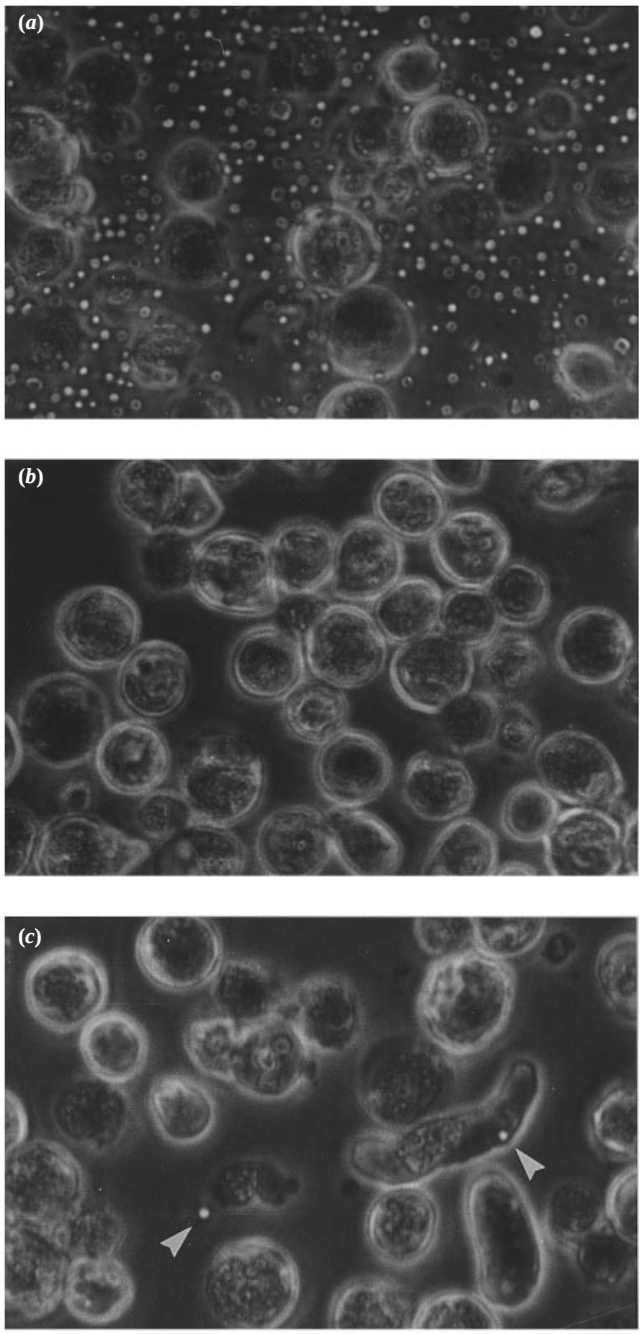

Fig. 6. Phase-contrast images of Sf21 cells infected with (*a*) the recombinant AcMO16, expressing AcMNPV P10, (*b*) the *p10* deletion mutant AcMO21 or (*c*) recombinant AcMVO7, expressing BusuNPV P10 at 5 days p.i. Arrows indicate polyhedra released from AcMVO7-infected cells.

Discussion

P10 proteins accumulate to high levels at the very late stage of an MNPV infection. In general, the amino acid sequence homology among the P10 proteins is low, as the consequence of highly diverged nucleotide sequences. This precludes the detection of *p10* in other baculovirus genomes by nucleic acid hybridization. In the genomes of AcMNPV, CfMNPV, OpMNPV, BmNPV and SeMNPV (Liu *et al.*, 1986; Bicknell *et al.*, 1987; Zuidema *et al.*, 1993; Wilson *et al.*, 1995; Palhan & Gopinathan, 1996; Poloumienko & Krell, 1997) the *p10* gene is preceded by the *p26* gene. The conserved gene arrangement of *p26* and *p10* was used to identify the *p10* gene of an SNPV, namely that of BusuNPV. Random sequence analysis of restriction fragments of BusuNPV DNA led to the identification of *p26*, which is located on the *Hin*dIII-D fragment. Sequencing downstream of *p26* revealed an ORF of 282 nt potentially encoding a protein with features characteristic for a P10 protein: N-terminal heptad-repeat motifs, a proline-rich domain and a positively charged C-terminal domain (see review by van Oers & Vlak, 1997). This strongly suggests that this ORF encodes a P10 homologue. This is the first report of a *p10* gene in an SNPV and it may imply that such a gene is preserved in all NPVs.

Downstream of the *p10* gene the genomic map of BusuNPV is different from MNPVs and shows an ORF homologous to the *odvp-6e* gene of OpMNPV and *Cydia pomonella* granulovirus (Theilmann *et al.*, 1996). In the genomes of AcMNPV, BmNPV, CfMNPV, OpMNPV and SeMNPV, the *p74* gene has been found at this position (Kuzio *et al.*, 1989; Leisy *et al.*, 1986; Hill *et al.*, 1993; Zuidema *et al.*, 1993; Palhan & Gopinathan, 1996).

The putative BusuNPV P10 protein formed fibrillar structures when expressed under control of the AcMNPV *p10* promoter in recombinant AcMVO7. This observation confirms that the 282 nt ORF encodes the BusuNPV P10 protein. The nuclear structures induced by this protein had a less fibrillar appearance than AcMNPV nuclear fibrillar structures. The fact that the nuclear structures interacted with electron-dense spacers, as do AcMNPV nuclear fibrillar structures, and that polyhedral envelopes were formed around AcMVO7 polyhedra, indicate that BusuNPV P10 assists in each aspect of AcMNPV polyhedron morphogenesis. Previously, it has been shown that SeMNPV P10 could replace its AcMNPV homologue in this function (van Oers *et al.*, 1994). The present result confirms the value of the 'swapping' assay for the functional analysis of putative P10 proteins.

In AcMNPV infections P10 is responsible for the disintegration of the nuclei at the final stage of infection and thus for the dissemination of separate polyhedra into the environment (Williams *et al.*, 1989; van Oers *et al.*, 1993). BusuNPV P10 protein released very few polyhedra when present in an AcMNPV environment (Fig. 6*c*). This parallels a previous observation (van Oers *et al.*, 1994) that SeMNPV P10 protein could not perform this function when present in an AcMNPV environment, even though the cells were permissive to SeMNPV. On the other hand, SeMNPV P10 was perfectly able to release polyhedra from the same cells when they were infected with SeMNPV (van Oers *et al.*, 1994). The results obtained here further support the view that P10 proteins show

specificity in performing this function and that other viral factors seem to contribute to this process. However, we cannot rule out the possibility that the amount of BusuNPV P10 protein produced during infection with the recombinant AcMVO7 is insufficient to achieve efficient disintegration of the cell nuclei.

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