FUNCTIONAL ANALYSIS OF THE BACULOVIRUS 10 KILODALTON PROTEIN

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STELLINGEN

1. De kerndesintegratie-functie van het 10 kilodalton eiwit van *Autographa californica* nuclear polyhedrosis virus (AcMNPV) ligt tussen aminozuur 52 en 70.

   Dit proefschrift.
   Williams *et al.* (1989). A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *Journal of General Virology* 70: 187-202.
   Hu *et al.* (1994). The p10 gene of natural isolates of *Bombyx mori* encodes a truncated protein with molecular weight of 7,700 dalton. *Journal of General Virology* 75, 000-000.

2. Het baculovirus 10 kilodalton eiwit is niet betrokken bij het verslijmen van geïnfecteerde rupsen.

   Dit proefschrift.

3. De aminozuurvolgorde PKCKCYKKIK in het AcMNPV helicase (p143) wordt door Lu en Carstens ten onrechte aangemerkt als kern-importsignaal.

   Lu, A. and Carstens, E.B. (1991). Nucleotide sequence of a gene essential for viral DNA replication in the baculovirus *Autographa californica* nuclear polyhedrosis virus. *Virology* 181, 336-347.

4. De "South-Western blot-methode" gebruikt door McClintock *et al.* om DNA bindende eiwitten op te sporen levert mogelijk irrelevante resultaten op, doordat de eiwitten in een gedenatureerde staat verkeren.

   Bowen *et al.* (1980). The detection of DNA-binding proteins by protein blotting. *Nucleic Acids Research* 8, 1.
   McClintock *et al.* (1991). DNA-binding proteins of baculovirus-infected cells during permissive and semipermissive replication. *Virus Research* 20, 133-145.

5. De methode gebruikt door Peakman *et al.* om recombinante baculovirussen op te sporen is verre van ideaal vanwege de hoge expressie van het reporter-gen.

   Peakman *et al.* (1992). Highly efficient generation of recombinant baculoviruses by enzymatically mediated site-specific *in vitro* recombination. *Nucleic Acids Research* 20, 495-500.

6. De expressie van het NIb-gen moet eerst worden aangetoond in transgene tabaksplanten alvorens geconcludeerd mag worden dat het replicase bescherming biedt tegen aardappelvirus Y.

> Audy *et al.* (1994). Replicase-mediated resistance to potato virus Y in transgenic tobacco. *Molecular Plant-Microbe Interactions* 7, 15-22.

7. Toepassing van het orale poliovaccin in gemeenschappen, waar slechts enkele individuen geïmmuniseerd zijn, vereist strenge hygiëne in verband met de instabiliteit van het geattenueerde virus in het darmkanaal.

> Macadam *et al.* (1993). Genetic basis of attenuation of the Sabin type 2 vaccine strain of poliovirus in primates. *Virology* 192, 18-26.

8. Gezien het verschil in salaris tussen hoogleraren en aio's is het niet verwonderlijk, dat er ook bijklussende aio's bestaan.

9. Het is zeer voorbarig te concluderen, dat 'yellow head disease', een ziekte bij garnalen in Thailand, veroorzaakt wordt door een baculovirus.

> Boonyaratpalin *et al.* (1993). Non-occluded baculo-like virus, the causative agent of yellow head disease in the black tiger schrimp (*Peneaus monodon*). *Gyobo Kenkyo* 28, 103-109.

10. De term "survival tours" lijkt verkeerd gekozen, omdat dergelijke avonturen er meestal niet op gericht zijn natuurgebieden te laten overleven.

Stellingen behorende bij het proefschrift

Functional analysis of the baculovirus 10 kilodalton protein

door Monique M. van Oers, te verdedigen op 8 juni 1994.

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CONTENTS

Chapter 1

GENERAL INTRODUCTION

INTRODUCTION

Baculoviruses are infectious agents that cause fatal disease in arthropods, predominantly insects of the order *Lepidoptera*. Over 400 species of this order are known as hosts for these viruses although a single virus isolate infects only one or a few related lepidopteran species. In addition, these viruses have been reported from species of the orders *Hymenoptera*, *Diptera*, *Coleoptera*, *Trichoptera*, *Thysanura* and *Homoptera*, as well as in several members of the orders *Crustacea* and *Arachnida* (Martignoni and Iwai, 1986). Baculovirus infections were first described in silkworms, where viral infection had devastating effects on the silk culture with large economic impact (see Benz, 1986, for a historical review). Since baculoviruses regulate the size of insect populations in nature and since they have a restricted host range, considerable attention has been paid to their potential as biological pesticide (reviewed by Martignoni and Iwai, 1986). A decade ago the interest in baculoviruses and their molecular biology was fueled by the observation, that they could be exploited for the efficient expression of heterologous genes. The high levels of expression and the ability of the system to modify the expressed proteins post-translationally, together with the inherent safety for humans, make this system a very attractive alternative to other higher-eukaryotic expression systems (e.g. vaccinia). Baculoviruses thus have a high profile not only in insect control as alternative to chemicals, but also as producer of proteins for fundamental research as well as for medical and veterinary practices.

The broad interest in baculoviruses is reflected in the large number of review papers and books that have been published in recent years: for instance on the general biology and ultrastructure (Granados and Federici, 1986; Adams and Bonami, 1991), the gene regulation and molecular biology (Friesen and Miller, 1986; Blissard and Rohrmann, 1990), their use as insecticides (Martignoni and Iwai, 1986) and their broad applications as expression vectors (Luckow and Summers, 1988; Miller, 1988; Maeda, 1989; Vlak

and Keus, 1990). Various manuals for experimental procedures contain a wealth of information (Summers and Smith, 1987; King and Possee, 1992; O'Reilly *et al.*, 1992).

TAXONOMY

The *Baculoviridae* are a family of rod-shaped (baculum=rod), enveloped, double-stranded DNA viruses with a circular, supercoiled genome varying in size between 90 and 230 kilobase pairs (kbp) (Francki *et al.*, 1991). Until recently, the family was subdivided in two subfamilies: *Eubaculovirinae* and *Nudibaculovirinae*. The *Eubaculovirinae* comprised the genera nuclear polyhedrosis virus (NPV) and granulosis virus (GV), which occlude their progeny virions into proteinaceous bodies, called polyhedra (NPVs) or granula (GVs). The subfamily *Nudibaculovirinae* contains only the genus non-occluded baculovirus (NOB). The subdivision of NPVs in multiple (MNPV) and single (SNPV) nucleocapsid forms, reflecting the number of nucleocapsids packaged within one viral envelope, was not based on phylogenetic evidence but on morphology. However, this feature of the virus can be useful in discriminating two virus species isolated from the same host. The classification of baculoviruses was changed recently, according to the proposals of the International Committee on Taxonomy of Viruses (22nd triennial meeting of the Executive Committee of ICTV, August 1993, Glasgow). It was decided to divide the family *Baculoviridae* into two genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV), thereby eliminating the subfamilies mentioned above.

In the experiments described in this thesis two NPV species were used. One was isolated from the alfalfa looper *Autographa californica* (AcMNPV). The AcMNPV prototype E2 (Vail *et al.*, 1971) is nowadays the most extensively studied baculovirus. The second species was isolated from the beet army worm, *Spodoptera exigua* (SeMNPV; Hunter and Hall, 1968a, b). Other NPV species relevant to this thesis are *Bombyx mori* NPV (BmNPV), *Orgyia pseudotsugata* MNPV (OpMNPV), *Choristoneura fumiferana* MNPV (CfMNPV) and *Perina nuda* MNPV (PnMNPV).

EPIDEMIOLOGY, PATHOLOGY AND BIOLOGY

The vertical transmission of baculoviruses in insect populations (i.e. from one generation to the next) occurs mainly at the time of oviposition by direct contamination of the eggs or the foliage in the vicinity of the neonatants by infected or surface-contaminated adults. Horizontal dissemination within the insect population (between individuals of the same generation) is caused directly by infected insects, by parasites, predator insects and sarcophageous flies. Mammals and birds, although not sensitive to a baculovirus infection, may help to disperse the virus, since the virus survives in the digestory tract of these animals. Abiotic factors involved in dissemination are gravity, rain or irrigation water, wind, carrying contaminated dust or aerosols, cultivation practices and marketing. The soil often functions as a reservoir for polyhedra (reviewed by Hostetter and Bell, 1985).

The external signs of infection of a lepidopteran larvae with a baculovirus first occur several days after ingestion of the virus and are marked by a reduced appetite, slow locomotion, retarded growth and an increased incidence of secondary infections (bacteria and fungi). Then the larvae stop feeding and finally hang by their prolegs from leaves and branches or lay in the soil. Death occurs accompanied by the liquefaction of internal tissues and the darkening of the cuticle at 3 to 8 days after the initial signs. The cuticle and epidermis often rupture, releasing masses of polyhedra (Tanada and Hess, 1984; Granados and Williams, 1986).

After ingestion, the polyhedra readily dissolve in highly alkaline lepidopteran midgut, releasing polyhedron-derived virions (PDVs), that start a primary infection in columnar cells of the midgut (Harrap *et al.*, 1970). The infection is a temporarily regulated, biphasic process. In the first phase progeny virus is produced by budding through the cytoplasmic membrane and the basal laminae into the hemocoel. These extracellular-viruses (ECVs) cause secondary infections in other tissues such as fat body, blood cells, hypodermis and trachea. Cells of other tissues such as muscles, nerves and glands are also susceptible in some cases. In the second phase of infection the nuclei become packed with polyhedra, which occlude many virions (Tanada and Hess, 1984; Granados and Williams, 1986).

The two virion types, ECV and PDV, contain the same genetic information, but differ substantially in morphology (e.g. a loosely attached versus a tight envelope;

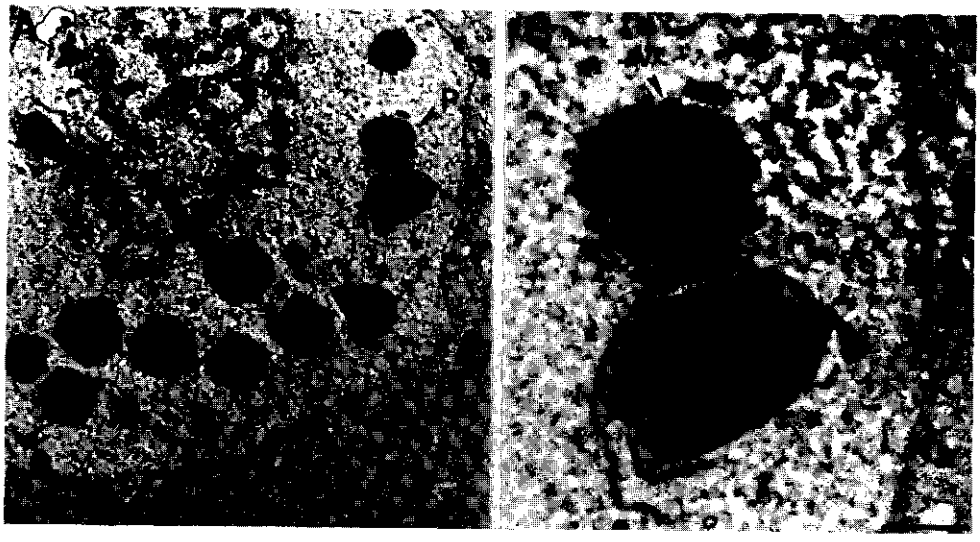

Figure 1.1. A) Electron micrograph of a thin section of a *Spodoptera frugiperda* cell infected with AcMNPV at 48 h post infection. B) Enlargement of a fibrillar structure and a polyhedron in the nucleus. F: fibrillar structure, P: polyhedron, ES: electron-dense spacer, VS: virogenic stroma, V: virus particle. Bar markers represent 1 μm (A) and 0.5 μm (B).

Federici, 1986) and protein composition. The 64 kilodalton (kDa) glycoprotein (gp64) for instance is characteristic for ECVs. ECVs and PDVs vary considerably in tissue specificity: ECVs are highly infectious for hemocytes, tissues adjacent to the hemocoel like the fat body, and *in vitro* for cultured insect cells. PDVs are most infectious for columnar midgut cells (Volkman *et al.*, 1976; Volkman and Summers, 1977).

CYTOPATHOLOGY

The cytopathology of baculoviruses has been studied both in larvae and in cultured lepidopteran cells. Cells in culture have the advantage, that the development of a baculovirus infection can be synchronized and that they can be visualized directly by light microscopy. The first cytopathologic effect observed in cells infected by an NPV is the enlargement of the nucleus. The central area of the nucleus contains Feulgen-positive material, called virogenic stroma. Later in infection growing polyhedra are observed in a

ring surrounding the virogenic stroma and afterward the nucleus becomes packed with polyhedra (Fig. 1.1. A; reviewed by Granados and Williams, 1986). Finally the infected cell lyses, thereby liberating the polyhedra from the nucleus. The polyhedra are surrounded by an electron-dense layer, called the polyhedral envelope or calyx. It is mainly composed of carbohydrate (Minion *et al.*, 1979), but it also contains a viral encoded protein, the polyhedral envelope protein (Whitt and Manning, 1988; Gombart *et al.*, 1989), which is essential for envelope formation (Zuidema *et al.*, 1989).

Another late effect is the formation of fibrillar structures in the nucleus and the cytoplasm of infected cells (Fig. 1.1 B). These fibrillar structures have been denoted alternatively as fibrous material (Summers and Arnott, 1969), fibrous sheets (MacKinnon *et al.*, 1974) or fibrous bodies (Tanada and Hess, 1984) and as a fibrous-like network (Knudson and Harrap, 1976). Fibrous material was also reported for *Trichoplusia ni* cells infected with a GV (Summers and Arnott, 1969). In NPVs the fibrillar structures in the nucleus are associated with multilamellar structures, referred to as electron-dense spacers (Summers and Arnott, 1969; MacKinnon *et al.*, 1974) or dense laminar profiles (Tanada and Hess, 1984). Occasionally, fibrillar structures are observed in close contact with polyhedral envelopes.

THE GENOMIC ORGANIZATION OF ACMNPV

The genome of AcMNPV is the best studied genome and is approximately 130 kbp in size (Cochran *et al.*, 1982). Physical maps of several strains of AcMNPV have been made (Smith and Summers, 1979; Cochran *et al.*, 1982), and a consensus orientation of the genome was proposed (Vlak and Smith, 1982). In the years following this pioneer work, the regulation of AcMNPV gene expression has been studied in detail. Many genes have been detected and characterized, and the genome has recently been completely sequenced (Possee, personal communication). In a recent review all the genes that are mapped up to now on the 131 kbp genome are described (Kool and Vlak, 1993). The genome also contains several highly repetitive (hr) sequences (Cochran and Faulkner, 1983), that serve as enhancers for transcription (Guarino *et al.*, 1986; Guarino and Summers, 1986) and possibly as origins of replication (Pearson *et al.*, 1992; Kool *et al.*, 1993).

The general model for baculovirus gene expression is an ordered cascade, in which each step or phase is dependent on previous events (reviewed by Friesen and Miller, 1986). Generally, genes are divided in early and late genes, depending on whether their

onset precedes DNA replication or not. Early genes are usually subdivided in immediate early or α-genes and delayed early or ß-genes. In *Spodoptera frugiperda* cells infected with AcMNPV immediate early gene expression occurs at 0-4 h post infection (p.i.) and is regulated by host cell factors. Delayed early gene expression (5-7 h p.i.) is dependent on or at least strongly stimulated by immediate early gene products. The late or γ-genes are most active directly after the onset of DNA replication, but the levels of expression decline at later times after infection. In this period (8-18 h p.i.) most of the DNA replication occurs and ECVs are produced. Unique for baculovirus gene expression is the existence of a fourth step in the cascade. This step involves the very late, hyperexpressed or δ-genes, which remain active until cell death, leading to accumulation of their gene products. These include the polyhedrin protein (29 Kda), which forms the crystalline matrix of polyhedra, and an approximately 10 Kda protein, which is generally denoted p10, and which is located in the fibrillar structures in the nucleus and cytoplasm of infected cells (Van der Wilk *et al.*, 1987; Quant-Russell *et al.*, 1987).

Early gene products are essential for DNA synthesis and for the shift to the late phase (Gordon and Carstens, 1984). The switch to late gene expression is gradual and the onset coincides well with the onset of DNA replication. Early viral RNA is synthesized by host RNA polymerase II, while late viral RNA synthesis is performed by a newly made α-amanitine-resistant RNA polymerase, not found in uninfected cells (Grula *et al.*, 1981; Fuchs *et al.*, 1983; Huh and Weaver, 1990). During infection the expression of host cell mRNAs and proteins gradually decreases and at later times only the synthesis of infected cell specific proteins prevails (Vlak *et al.*, 1981; Ooi and Miller, 1988). The transition to the late stage and the shut down of host protein synthesis is blocked when DNA-synthesis is prevented (Ooi and Miller, 1988).

There is no clustering of genes according to their time of expression in the AcMNPV genome (Adang and Miller, 1982; Esche *et al.*, 1982; Lübbert and Doerfler, 1984). Gene expression involves individual transcription units composed of temporarily-regulated, overlapping RNA transcripts, with either common 3' or 5' ends. The occurrence of overlapping transcripts with common 3' ends may be regulated by a process called transcriptional interference or promoter occlusion, a process in which transcription initiated at a more upstream promoter prevents initiation of a downstream promoter. This type of regulation leads to the rise of longer transcripts with time (Friesen and Miller, 1985). Overlapping RNAs with opposite polarity (antisense RNA) may play a role in gene regulation (Hardin and Weaver, 1990; Ooi and Miller, 1990). Transcripts with common

5' ends may result from read through of polyadenylation signals. In this case the shortest transcript is the most abundant one. This type of transcription was found for both the polyhedrin and p10 gene (see below). In general AcMNPV transcripts are unspliced (Lübbert and Doerfler, 1984). Splicing was only reported for immediate early gene 1 (IE-1) transcripts (Chisholm and Henner, 1988). It has been suggested that overlapping transcripts might provide a variety that might otherwise be achieved by splicing (Lübbert and Doerfler, 1984).

THE VERY LATE GENES

The polyhedrin genes of many NPVs have been studied intensively (reviewed by Vlak and Rohrmann, 1985; Rohrmann, 1986; Zanotto *et al.*, 1993). The function of polyhedrin is the occlusion of virions to protect against proteolysis and environmental decay. In contrast, for p10 genes and their gene products data are scarce and the function of p10 protein is not known. The functional analysis of baculovirus p10 proteins is the central subject of this thesis.

The AcMNPV p10 protein was announced as a 7.2 kDa (Adang and Miller, 1982), 7.5 kDa (Vlak *et al.*, 1981) or 8 kDa protein (Rohel *et al.*, 1983) based upon its behavior upon SDS-polyacrylamide gel electrophoresis. For OpMNPV a 14 Kda protein was reported (Quant-Russell *et al.*, 1987). The name 10K protein (p10) was given by (Smith *et al.*, 1982; 1983a) and has been confirmed by the predicted molecular weight, calculated from sequence data (Kuzio *et al.*, 1984), which is approximately 10 kilodalton. Pulse labelling experiments showed that p10 protein is first detected at 12-15 h p.i. and is synthesized till at least 99 h p.i. (Rohel *et al.*, 1983; Smith *et al.*, 1983a).

The genetic organization of a segment of the AcMNPV genome containing the p10 gene is depicted in Fig. 1.2. The AcMNPV p10 protein is encoded by an uninterrupted single-copy gene, mapped by Northern blotting, *in vitro* translation and CDNA analysis to the *Pst*I-B and the *Eco*RI-P fragment on the *Hin*dIII-P/Q boundary between map units 87.35 and 89.55 (Adang and Miller, 1982; Rohel *et al.*, 1983; Smith *et al.*, 1983a; Rankin *et al.*, 1986). The open reading frame (ORF) of 282 nucleotides (nt) encodes a protein of 94 amino acids and is preceded by an AT-rich region, whereas polyadenylation signals are found downstream of the ORF (Kuzio *et al.*, 1984). With the AcMNPV p10 sequence as a probe the OpMNPV p10 gene was found and characterized (Leisy *et al.*, 1986a). In OpMNPV the p10 ORF is 276 nt and encodes a protein of 92 amino acids. The

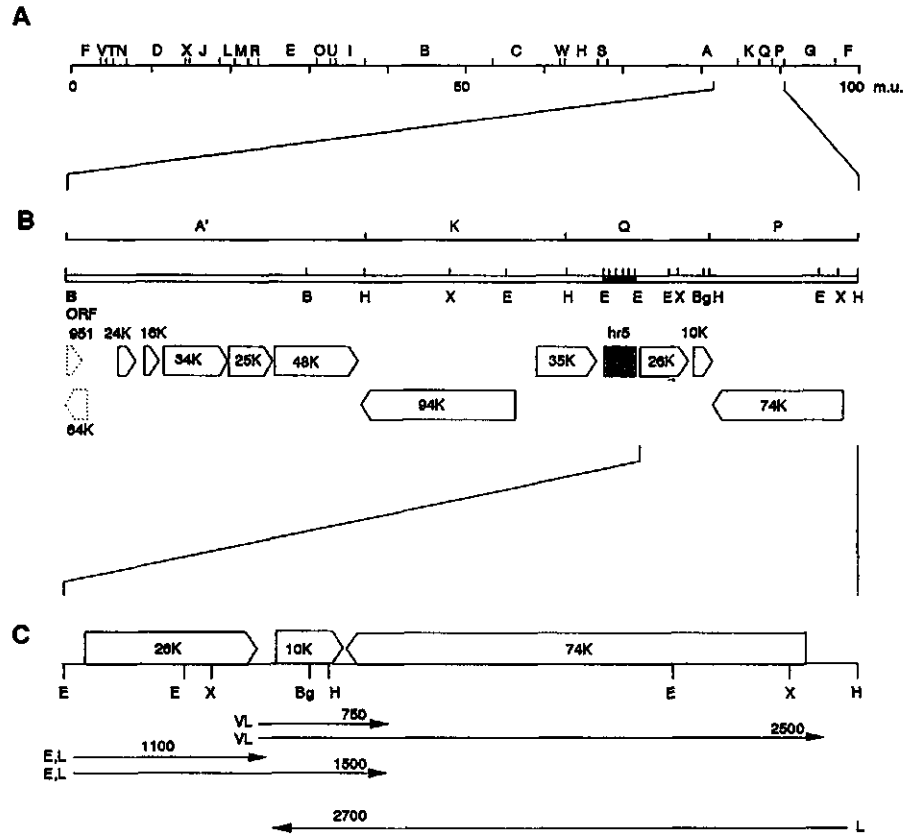

Figure 1.2. A) Linear physical map of the AcMNPV genome for restriction endonuclease *Hin*dIII. The fragments are alphabetical numbered according to their size. One map unit (m.u.) represents 1.3 kbp. B) Physical map and genomic organization of an 11.8 kbp segment of the AcMNPV genome, encompassing part of the *Hin*dIII A fragment (A') and the complete *Hin*dIII K, Q and P fragments. ORFs are indicated by open arrows. Dotted arrows represent ORFs that extend beyond the depicted region. The 64K ORF encodes the budded virion specific glycoprotein gp64. The ORFs 24K, 16K, 34K, 25K and 48K are also known as ORF I to V. ORF 34K encodes the polyhedral envelope or calyx protein (pp34). C) Transcripts mapped in the AcMNPV p10 gene region. Transcripts are represented as arrows and the sizes are indicated in nucleotides. E stands for early, L for late and VL for very late transcript. Restriction sites are indicated as B: *Bam*HI, Bg: *Bgl*II, E: *Eco*RI, H: *Hin*dIII and X: *Xho*I. The data for this figure were extracted from the review by Kool and Vlak (1993) and references therein.

p10 coding sequences of AcMNPV and OpMNPV share 54% nucleotide sequence homology and 41% amino acid sequence homology (Leisy *et al.*, 1986a). Most homology was observed at the amino-terminus of the protein. Both p10 proteins do not contain any methionine, cysteine, tyrosine, tryptophan and histidine residues. They are however rich in serine and threonine, amino acids that can be involved phosphorylation, and in proline and lysine. The polyhedrin genes of AcMNPV and OpMNPV were very similar, showing 80% and 90% homology at the nucleotide and amino acid sequence level, respectively (Leisy *et al.*, 1986b), suggesting common ancestry.

In both AcMNPV (Fig. 1.2 B) and OpMNPV upstream of the p10 gene an ORF, denoted as ORF p26, was found with the same polarity as p10 (Liu *et al.*, 1986; Bicknell *et al.*, 1987). A function has not yet been assigned to the p26 gene. Downstream of the p10 gene the p74 ORF is located, which has an opposite polarity. P74 is a late gene and is essential for infection of larvae with PDVs and hence a virulence factor (Kuzio *et al.*, 1989).

The AcMNPV p10 region is transcribed in four overlapping transcripts running in the same direction (Fig. 1.2 C), as determined by Northern blotting, S1 nuclease mapping and primer extension experiments (Rohel *et al.*, 1983; Friesen and Miller, 1985; Rankin *et al.*, 1986; Kuzio *et al.*, 1989). Two very late poly(A)$^+$ RNA transcripts of 750 and 2500 nt, first detected 10-12 h p.i., rapidly accumulate and continue to be formed till at least 60 h p.i. These 750 and 2500 nt transcripts have common 5' ends that map at position -69/-70 relative to the ATG start codon of the p10 ORF. Two other poly(A)$^+$ transcripts (1100 and 1500 nt) are maximally expressed 12 h p.i. and these two RNAs also have common 5' ends, which map just upstream of the start of the p26 ORF. The late 1500 and very late 750 nt transcripts share their 3' ends. The 2500 nt band appears to have two different 3' ends, one of which shows microheterogeneity. The 750, 1500 and 2500 nt transcripts comprise the complete p10 coding sequence. The 1100 and 1500 nt transcripts overlap with the p26 ORF. Polyadenylation signals (AATAAA) are found for the 750 and 1500 nt, and for the 2500 nt transcripts. A slightly modified signal (ATTAAA) is present for the 1100 nt transcript. A 2700 nt transcript of the p74 gene also maps in the p10 region, but in the other direction. The 750 nt MRNA was translated *in vitro* into a methionine deficient protein of 8 kDa (Rohel *et al.*, 1983). A similar pattern of overlapping transcripts was found for the polyhedrin gene (Friesen and Miller, 1985; Howard *et al.*, 1986). Polyhedrin and p10 gene transcripts appear in approximately equal amounts and together accumulate to more than 90% of the total poly (A)$^+$ viral RNA very

late after infection (Smith *et al.*, 1983a). The polyhedrin and p10 genes of *Orgyia pseudotsugata* MNPV (OpMNPV) also showed a remarkably similar transcription pattern (Leisy *et al.*, 1986a; 1986b). Very late gene transcription thus appears to occur according to a general pattern in baculoviruses, showing transcripts with common 5' ends of which the shortest ones are also the most abundant ones. The 5' end of the major p10 messengers of both AcMNPV and OpMNPV mapped within the sequence ATAAG (Leisy *et al.*, 1986a; Kuzio *et al.*, 1986; Rankin *et al.*, 1986), which is part of a 12 nucleotide consensus sequence found near the mRNA cap-site of all polyhedrin, granulin and p10 genes analysed thus far, (A/T)ATAAGNANT(T/A)T, also known as the Rohrmann-box (Rohrmann, 1986). An other similarity is the AT-richness of the non-translated leader sequence.

Several transient expression experiments were performed to define the AcMNPV p10 promoter, using chloramphenicol acetyl transferase as reporter for gene expression. The p10 promoter was only active when plasmids were transfected into infected insect cells, indicating that the activity is either directly or indirectly dependent on other viral gene products (Knebel *et al.*, 1985; Weyer and Possee, 1988). Maximum p10 expression requires the integrity of the complete untranslated leader sequence plus some additional upstream nucleotides (positions -72 to +1) (Weyer and Possee, 1988, 1989; Qin *et al.*, 1989). These studies also showed that the p10 promoter is relatively small, and does not extend the mRNA cap-site by more than several nucleotides. However, when the ATAAG sequence is mutated, expression levels drop sharply. Also the *in vitro* methylation of a 5' CCGG 3'motif in the p10 promoter region inactivated the p10 promoter (Knebel *et al.*, 1985).

FUNCTIONAL STUDIES ON THE P10 PROTEIN

The p10 protein is not associated with ECVs, PDVs or polyhedra, although some p10 copurifies with polyhedra (Vlak *et al.*, 1981). Immunofluorescence studies and immunogold labelling experiments showed that p10 protein was associated with fibrillar structures formed in the nucleus and cytoplasm of AcMNPV or OpMNPV-infected cells (Quant-Russell *et al.*, 1987; Van der Wilk *et al.*, 1987). One of the monoclonal antibodies raised against the OpMNPV p10 protein showed a strong reaction with cytoskeletal structures in uninfected cells. This might reflect structural homology between p10 and a cytoskeletal component (Quant-Russell *et al.*, 1987). Immunogold labelling also

showed that polyhedrin was not present in these structures. Furthermore, mutants without an intact polyhedrin gene were still able to form fibrillar structures (van der Wilk, 1987). These results led to the rejection of the hypothesis of Chung *et al.* (1980), that fibrillar structures were deposits of precondensed polyhedrin.

Early functional studies concerning the p10 protein were hampered by the absence of an appropriate screening system for the selection of p10 mutant viruses. To circumvent this difficulty the p10 gene was inactivated by inserting marker genes into the p10 coding sequence, such as the phosphotransferase gene, conferring resistence against the antibiotic geneticin (G418) to the recombinant viruses (Gonnet and Devauchelle, 1987; Croizier *et al.*, 1987) or the enzymatic marker gene lacZ (Vlak *et al.*, 1988; Williams *et al.*, 1989). These studies showed that p10 was dispensable for viral infection, but that it was essential for the formation of fibrillar structures.

The in-frame insertion of the lacZ sequence led to the expression of a fusion protein, containing the amino-terminal 52 amino acids of p10 followed by the ß-galactosidase sequence (Vlak *et al.*, 1988; Williams *et al.*, 1989). The fusion protein formed granular structures in the nucleus of recombinant-infected cells, that reacted with antiserum against ß-galactosidase (Vlak *et al.*, 1988). In contrast to wild type infected cells, cells infected with p10 recombinants with either in- or out-frame lacZ insertions did not release polyhedra (Williams *et al.*, 1989), indicating that p10 might have a function in cell lysis. The disruption of the p10 gene by the lacZ sequence prevented the formation of polyhedral envelopes. A p10 deletion mutant resulted in fragile polyhedra with a loosely attached envelope (Williams *et al.*, 1989), indicating that p10 might have a function in the assembly of polyhedral envelopes.

The results obtained with the lacZ insertion mutants were rather confusing, since they were not entirely consistent between the two studies. Vlak *et al.* (1988) reported that electron-dense spacers were absent from cells infected with the p10-lacZ fusion mutant, in contrast to Williams *et al.* (1989), who observed normal spacers. The reason for this discrepancy is not clear, but could be the introduction of an additional mutation some where else in the genome. Another possibility is that this ambiguity is due to the minor differences in the cloning strategy, which resulted in small alterations at the fusion point. The introduction of a proline residue at the fusion point as reported in one study (Williams *et al.*, 1989) may have a drastic influence on the conformation of the fusion protein. Furthermore, the remaining part of p10 in such a fusion protein might be affected in its biological function by the ß-galactosidase moiety. Therefore, the analysis of mutant

viruses having simple p10 deletions or point mutations may be more convenient to study the function of p10 protein. The availabilty of a new p10-promoter based transfer vector system (Vlak *et al.*, 1990) provides an excellent opportunity to circumvent the disadvantages of fusion proteins. After cloning of a modified p10-coding sequence in the appropriate transfer plasmid, mutant viruses can be obtained by recombination and these mutants can be easily selected by virtue of their ß-galactosidase expression (see below).

THE BACULOVIRUS EXPRESSION SYSTEM

Both very late baculovirus genes contain strong promoters and the high gene dose present late in infection may also contribute to the synthesis of massive amounts of polyhedrin and p10. This high level of expression, together with the fact that both polyhedrin and p10 are not essential for viral replication *in vitro* (Smith *et al.*, 1983b; Gonnet and Devauchelle, 1987; Vlak *et al.*, 1988), made both very late promoters available for foreign gene expression without requiring helper virus. This resulted in the development of baculovirus expression vectors, which were mainly based upon polyhedrin promoter-driven expression. These vectors have been used extensively to express a variety of foreign genes of animal, viral, fungal, bacterial and plant origin (Luckow, 1991; O'Reilly *et al.*, 1992). The observation that the expressed proteins were amenable to post-translational modifications as in mammals, such as glycosylation, phosphorylation and myristillation, thereby producing functionally and antigenically active proteins, further improved the value of the system (Vlak and Keus, 1990).

The baculovirus expression system is based on the construction of recombinant viruses that contain a heterologous gene downstream of the polyhedrin or p10 promoter. The system based on the p10 promoter is depicted in Fig. 1.3. The first step in obtaining such a recombinant virus is the construction of a transfer plasmid, that replicates in *Escherichia coli*. It contains either the polyhedrin or p10 promoter followed by an insertion site for heterologous genes. Additionally, this transfer plasmid (or transfer vector) contains sequences homologous to the DNA sequences, that flank the polyhedrin or p10 gene in the wild type viral genome. Upon cotransfection of viral DNA and the transfer plasmid, recombinant progeny viruses are likely to arise, due to homologous recombination within the flanking regions. The percentage of recombinant viruses depends on the length of the flanking sequences and the size of the heterologous gene, and varies between 0.1 and 5% (Summers and Smith, 1987).

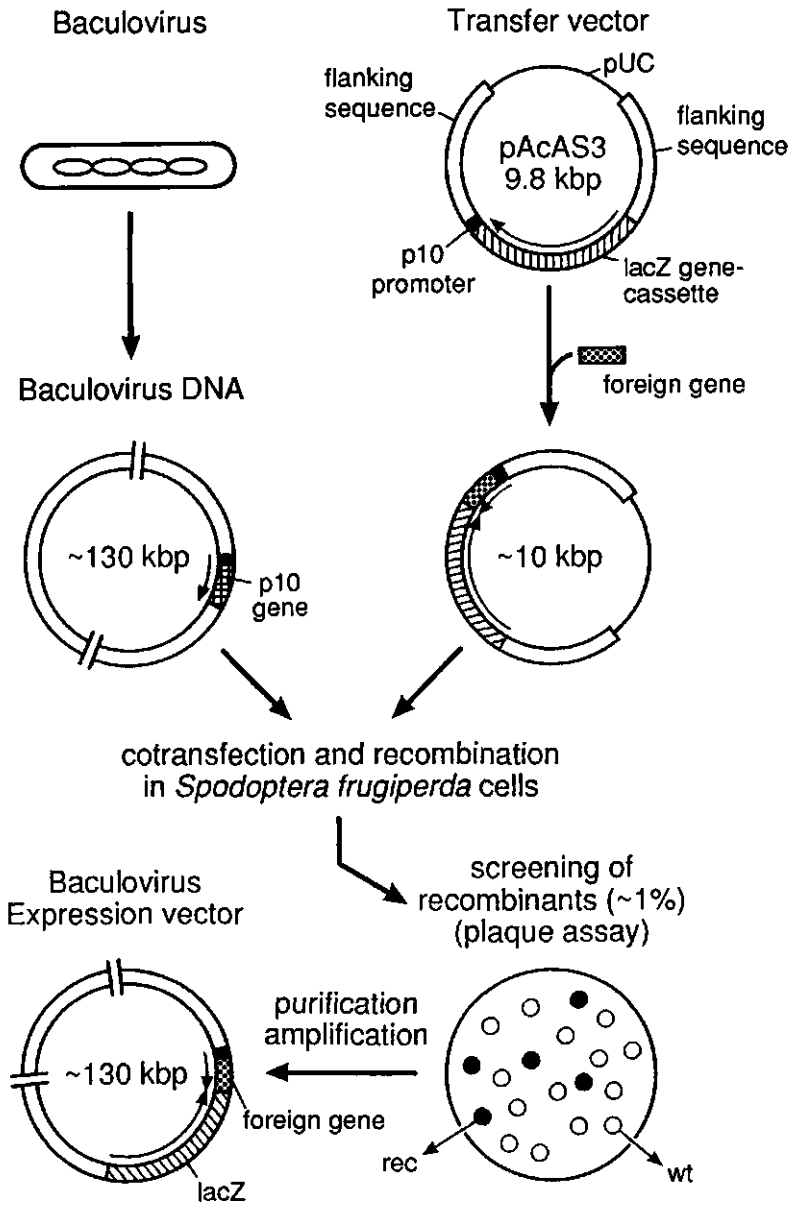

Figure 1.3. The construction of baculovirus expression vectors based upon the p10-replacement vector pAcAS3 (Vlak *et al.*, 1990). The lacZ gene cassette consists of the *D. melanogaster* heat shock 70 promoter (hsp), the *E. coli* lacZ sequence and the SV40 terminator sequence. The direction of transcription is indicated by arrows.

The next step is to select the recombinant viruses from the parental viruses. In the case of replacement in the polyhedrin locus the recombinant viral plaques have a polyhedron-minus phenotype, which can be seen easily by light microscopy. The main difficulty in obtaining p10-promoter based recombinants has been their selection, since recombinant viral plaques can not be discriminated light microscopically by virtue of their p10-minus phenotype. To overcome this problem, a novel type of transfer vector was designed (pAcAS3; Vlak *et al.*, 1990). It contains a reporter gene controlled by a constitual promoter, which is transferred into the genome together with the foreign gene (Fig. 1.3). The selection is based on the constituent expression of ß-galactosidase by recombinant viral plaques. This enables visual selection of recombinant viruses by virtue of the blue plaque phenotype upon the addition of the chromogenic substrate X-gal.

The p10 promoter may be preferred over the polyhedrin promoter when *in vivo* infectivity of the recombinant is required, for instance when employing recombinant baculoviruses for insect control. When both the polyhedrin and p10 promoter are used, simultaneous expression of two proteins can be achieved. An examples of this application is the concurrent expression of parvovirus capsid proteins (Brown *et al.*, 1991) or bluetongue reovirus proteins (Belyaev and Roy, 1993), leading to the assembly of parvovirus empty capsids and reovirus-like particles, respectively.

OUTLINE OF THE THESIS

Research on very late genes of nuclear polyhedrosis viruses has concentrated mainly on the exploitation of their promoters for the expression of heterologous genes. For this purpose the localization of the polyhedrin and p10 gene, and the analysis of their promoters are indispensable. Optimal levels of foreign gene expression are of course demanded and several determinants for high level gene expression have been reported (Matsuura *et al.*, 1987; Possee and Howard, 1987; Weyer and Possee, 1988, 1989; Qin *et al.*, 1989). In Chapter 2 the effect of the simultaneous activity of both very late promoters on the level of expression of the individual gene products is examined, by analysing recombinants with deletions in the polyhedrin gene.

The exploitation of the p10 locus for the expression of heterologous genes, as for instance insect hormone or toxin genes, needs understanding of the consequences for the infection process, the virulence and the persistance in the field. Therefor, knowledge of the function of p10 is highly relevant. The strategy adopted for the functional analysis of

p10 involves the construction and characterization of p10 mutants. However, with only the sequences of the closely related AcMNPV and OpMNPV p10 genes available, it is difficult to predict conserved amino acid sequences or distinct domains that may be essential for the functioning of p10. To obtain optimal profit from amino acid sequence data, the p10 gene of the distantly related baculovirus SeMNPV is characterized (Chapter 3).

On the basis of sequence comparisons several functional domains are postulated (Chapter 3 and 4) and AcMNPV p10 deletion mutants are constructed to analyse these putative domains further (Chapter 4). These mutants are caracterized in terms of fibrillar structure formation, release of polyhedra and presence of polyhedron envelopes and electron-dense spacers. An enzymatic test is used to determine whether p10 induces cell lysis (as proposed by Williams *et al.*, 1989) or nuclear disintegration at the final stage of infection. To analyse the specificity of p10 functions the p10 ORF of AcMNPV is replaced with the distantly related SeMNPV p10 ORF (Chapter 5). Experiments are performed to determine whether p10 proteins of different species can co-assemble into one fibrillar structure, and whether fibrillar structure formation and polyhedron release require specific interactions of p10 with host or viral factors. Chapter 6 describes the further analysis of the carboxy terminus of AcMNPV p10. This domain contains two serine residues that are prone to phosphorylation by cAMP-dependent protein kinase (Cheley *et al.*, 1992). These serines are replaced by alanine residues to examine whether phosphorylation at one of these serines is required for fibrillar structure formation. In Chapter 7 the experimental data are discussed and a model is proposed, postulating several functional domains on the AcMNPV p10 protein.

REFERENCES

Adang, M.J. and Miller, L.K. (1982). Molecular cloning of DNA complementary to mRNA of the baculovirus *Autographa californica* nuclear polyhedrosis virus: location and gene products of RNA transcripts. *Journal of Virology* 44: 782-793.

Adams, J.R. and Bonami, J.R., eds (1991). *Atlas of Invertebrate Pathology.* CRC-Press, Florida.

Belyaev, A.S and Roy, P. (1993). Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells. *Nucleic Acids research* 21: 1219-1223.

Benz, G.A. (1986). Introduction: Historical perspectives. In: *The biology of baculoviruses, volume I Biological properties and molecular biology* (R.R. Granados and B.A. Federici, eds) pp. 1-35, CRC-Press, Florida.

Bicknell, J.N., Leisy, D.J., Rohrmann, G.F. and Beaudreau, G.S. (1987). Comparison of the p26 gene region of two baculoviruses. *Virology* 161: 589-592.

Blissard, G.W. and Rohrmann, G.F. (1990). Baculovirus diversity and molecular biology. *Annual Review of Entomology* 35: 127-155.

Brown, C.S., Van Lent, J.W.M., Vlak, J.M. and Spaan, W.J.M. (1991). Assembly of empty capsids by using baculovirus recombinants expressing parvovirus B19 structural proteins. *Journal of Virology* 65: 2702-2706.

Cheley, S., Kosik, K.S., Paskevich, P., Bakalis, S. and Bayley, H. (1992). Phosphorylated baculovirus p10 is a heat-stable microtubule-associated protein associated with process formation in SF-9 cells. *Journal of Cell Science* 102:739-752.

Chisholm, G.E. and Henner, D.J. (1988). Multiple early transcripts and splicing of the *Autographa californica* nuclear polyhedrosis virus IE-1 gene. *Journal of Virology* 62: 3193-3200.

Chung, K.L., Brown, M. and Faulkner, P. (1980). Studies on the morphogenesis of polyhedral inclusion bodies of a baculovirus *Autographa californica* NPV. *Journal of General Virology* 46: 335-347.

Cochran, M.A., Carstens, E.B., Eaton, B.T. and Faulkner, P. (1982). Molecular cloning and physical mapping of restriction endonuclease fragments of *Autographa californica* nuclear polyhedrosis virus DNA. *Journal of Virology* 41: 940-946.

Cochran , M.A. and Faulkner, P. (1983). Location of homologous DNA sequences interspersed at five regions in the baculovirus AcMNPV genome. *Journal of Virology* 45: 961-970.

Croizier, G., Gonnet, P. and Devauchelle, G. (1987). Localisation cytologique de la protéine non structurale p10 du baculovirus de la polyédrose nucléaire du Lépidoptère *Galleria mellonella* L. *C.R. Acad. Sci. Paris, t. 305, Série III*, pp. 677-681.

Esche, H., Lübbert, H., Siegmann, B. and Doerfler, W. (1982). The translational map of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome. *The EMBO Journal* 1: 1629-1633.

Federici, B.A. (1986). Ultrastructure of baculoviruses. In: *The Biology of Baculoviruses, Volume 1. Biological Properties and Molecular Biology* (R.R. Granados and B.A. Federici, eds), pp. 61-88, CRC-Press, Florida.

Francki, R.I.B., Fauquet, C.M., Knudson, D. and Brown, F. (eds) (1991). Classification and nomenclature of viruses: Fifth Report of the International Committee on Taxonomy of Viruses. *Archives of Virology*: supplementum 2.

Friesen, P.D. and Miller, L.K. (1985). Temporal regulation of baculovirus RNA: Overlapping early and late transcripts. *Journal of Virology* 54: 392-400.

Friesen, P.D., and Miller, L.K. (1986). The regulation of baculovirus gene expression. In: *The Molecular Biology of Baculoviruses* (W. Doerfler and P. Böhm, eds). *Current Topics in Microbiology and Immunology* 131: 31-49.

Fuchs, Y.L., Woods, M.S. and Weaver, R.F. (1983). Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection: A novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. *Journal of Virology* 48: 641-646.

Gelernter, W.D. and Federici, B.A. (1986). Isolation, identification, and determination of virulence of a nuclear polyhedrosis virus from the beet army worm, *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environmental Entomology* 15: 240-245.

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

Gonnet, P. and Devauchelle, G. (1987). Obtention par recombinaison dans le gène du polypeptide p10 d'un baculovirus exprimant le gène de résistance à la néomycine dans le cellules d'insecte. *C.R. Acad. Sci. Paris, t. 305, Serie III*, pp. 111-114.

Gordon, J.D. and Carstens, E.B. (1984). Phenotypic characterization and physical mapping of a temperature sensitive mutant of *Autographa californica* nuclear polyhedrosis virus defective in DNA synthesis. *Virology* 138: 69-81.

Granados, R.R. and Federici, B.A., eds (1986). *The Biology of Baculoviruses, Volume 1. Biological Properties and Molecular Biology*, CRC-Press, Florida.

Granados, R.R. and Williams, K.A. (1986). In vivo infections and replication of baculoviruses. In: *The Biology of Baculoviruses, Volume 1. Biological Properties and Molecular Biology* (R.R. Granados and B.A. Federici eds), pp. 89-108, CRC-press, Florida.

Grula, M.A., Buller, P.L. and Weaver, R.F. (1981). α-Amanitin-resistent viral RNA synthesis in nuclei isolated from nuclear polyhedrosis virus-infected *Heliothis zea* larvae and *Spodoptera frugiperda* cells. *Journal of Virology* 38: 916-921.

Guarino, L.A. and Summers, M.D. (1986). Interspersed homologous DNA of *Autographa californica* nuclear polyhedrosis virus enhances delayed-early gene expression. *Journal of Virology* 60: 215-223.

Guarino, L.A., Gonzalez, M.A. and Summers, M.D. (1986). Complete sequence and enhancer function of the homologous DNA regions of *Autographa californica* nuclear polyhedrosis virus. *Journal of Virology* 60: 224-229.

Harrap, K.A. (1970). Cell infection by a nuclear polyhedrosis virus. *Virology* 42: 311-318.

Hardin, S.E. and Weaver, F. (1990). Overlapping transcripts mapping to the *Hin*dIII-F region of the *Autographa californica* nuclear polyhedrosis virus. *Journal of General Virology* 71: 225-229.

Hostetter, D.L. and Bell, M.R. (1985). Natural dissemination of baculoviruses in the environment. In: *Viral Insecticides for Biological Control* (K. Maramorosch and K.E. Sherman, eds), pp. 240-284.

Howard, S.C., Ayres, M.D. and Possee, R.D. (1986). Mapping the 5' end 3' ends of *Autographa californica* nuclear polyhedrosis virus polyhedrin mRNA. *Virus Research* 5: 109-119.

Huh, N.E. and Weaver, R.F. (1990). Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus. *Journal of General Virology* 71: 195-201.

Hunter, D.K. and Hall, I.M. (1968a). Pathogenicity of a nucleopolyhedrosis virus of the beet armyworm, *Spodoptera exigua*. *Journal of Invertebrate Pathology* 12: 83-85.

Hunter, D.K. and Hall, I.M. (1968b). Cytopathology of a nucleopolyhedrosis virus of the beet armyworm, *Spodoptera exigua*. *Journal of Invertebrate Pathology* 12: 93-97.

King, L.K. and Possee, R.D. (1992). *The Baculovirus Expression System. A Laboratory Guide*. Chapman and Hall, London.

Knebel, D., Lübbert, H. and Doerfler, W. (1985). The promoter of the late p10 gene in the insect nuclear polyhedrosis virus *Autographa californica*: activation by viral gene products and sensitivity to DNA methylation. *The EMBO Journal* 4: 1301-1306.

Knudson, D.L. and Harrap, K.A. (1976). Replication of a nuclear polyhedrosis virus in a continous cell culture of *Spodoptera frugiperda*: Microscopy study of the sequence of events of the virus infection. *Journal of Virology* 17: 254-268.

Kool, M., Van den Berg, P.M.M.M., Tramper, J., Goldbach. R.W. and Vlak, J.M. (1993). Location of two putative origins of DNA replication of *Autographa californica* nuclear polyhedrosis virus. *Virology* 192: 94-101.

Kool, M. and Vlak, J.M. (1993). The structural and functional organization of the *Autographa californica* nuclear polyhedrosis virus. *Archives of Virology* 130: 1-16 (plus updated version of December 1993).

Kuzio, J., Rohel, D.Z., Curry, C.J., Krebbs, A., Carstens, E.B. and Faulkner, P. (1984). Nucleotide sequence of the p10 gene of *Autographa californica* nuclear polyhedrosis virus. *Virology* 139: 414-418.

Kuzio, J., Jaques, R. and Faulkner, P. (1989). Identification of p74, a gene essential for virulence of baculovirus occlusion bodies. *Virology* 173: 759-763.

Leisy, D.J., Rohrmann, G.F., Nesson, M. and Beaudreau, G.S. (1986a). Nucleotide sequence and transcriptional mapping of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus p10 gene. *Virology* 153: 157-167.

Leisy, D.J., Rohrmann, G.F., and Beaudreau, G.S. (1986b). The nucleotide sequence of the polyhedrin gene region from the multicapsid baculovirus of *Orgyia pseudotsugata*. *Virology* 153: 280-288.

Liu, A., Qin, J., Rankin, C., Hardin, S.E. and Weaver, R.F. (1986). Nucleotide sequence of a portion of *Autographa californica* nuclear polyhedrosis virus genome containing the *Eco*RI site-rich region (hr5) and an open reading frame just 5' of the p10 gene. *Journal of General Virology* 67: 265-257.

Lübbert, H. and Doerfler, W. (1984). Mapping of early and late transcripts encoded by the *Autographa californca* nuclear polyhedrosis virus genome: Is viral RNA spliced? *Journal of Virology* 50: 497-506.

Luckow, V.A. and Summers, M.D. (1988). Signals important for high-level expression of foreign genes in *Autographa californica* nuclear polyhedrosis virus expression vectors. *Virology* 167: 56-71.

Luckow, V.A. (1991). Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In: *Recombinant DNA Technology and Applications* (A. Prokop, R.K. Bajpai and C.S. Ho, eds). pp. 97- 152, McGraw-Hill, Inc., New-York.

MacKinnon, E.A., Henderson, J.F., Stolz, D.B. and Faulkner, P. (1974). Morphogenesis of nuclear polyhedrosis virus under conditions of prolonged passage *in vitro*. *Journal of Ultrastructural Research* 49: 419-435.

Matsuura, Y., Possee, R.D., Overton, H.A. and Bishop, D.H.L. (1987). Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. *Journal of General Virology* 68: 1233-1250.

Maeda, S. (1989). Expression of foreign genes in insects using baculovirus vectors. *Annual Review of Entomology* 34: 351-372.

Martignoni, M.E. and Iwai, P.J. (1986). A Catalog of Viral Diseases of Insects, Mites and Ticks. *USDA Forest Service PNW-195*. Washington, DC: USPGO.

Miller, L.K. (1988). Baculoviruses as gene expression vectors. *Annual Review of Microbiology* 42: 177-199.

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

Ooi, B.G. and Miller, L.K. (1988). Regulation of host RNA levels during baculovirus infection. *Virology* 166: 515-523.

Ooi, B.G. and Miller, L.K. (1990). Transcription of the baculovirus polyhedrin gene reduces the levels of antisense transcript initiated downstream. *Journal of Virology* 64: 3126-3129.

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

Pearson, M., Bjornson, R., Pearson, G. and Rohrmann, G.F. (1992). The *Autographa californica* baculovirus genome: evidence for multiple replication origins. *Science* 257: 1382-1384.

Possee, R.D. and Howard, S.C. (1987). Analysis of the polyehedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus. *Nucleic Acids Research* 15: 10233-10248.

Quant-Russell, R.L., Pearson, M.N., Rohrmann, G.F. and Beaudreau, G.S. (1987). Characterization of baculovirus p10 synthesis using monoclonal antibodies. *Virology* 160: 9-19.

Qin, J., Liu, A. and Weaver, R.F. (1989). Studies on the control region of the p10 gene of the *Autographa californica* nuclear polyhedrosis virus. *Journal of General Virology* 70: 1273-1279.

Rankin, C., Ladin, B.F. and Weaver, R.F. (1986). Physical mapping of temporally regulated, overlapping transcripts in the region of the 10K protein gene in *Autographa californica* nuclear polyhedrosis virus. *Journal of Virology* 57: 18-27.

Roelvink, P.W., Van Meer, M.M.M., De Kort, C.A.D., Possee, R.D., Hammock, B.D. and Vlak, J.M. (1992). Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and p10 genes. *Journal of General Virology* 73: 1481-1489.

Rohel, D.Z., Cochran, M.A. and Faulkner, P. (1983). Characterization of two abundant mRNAs of *Autographa californica* nuclear polyhedrosis virus present late in infection. *Virology* 124: 357-365.

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

Smith, G.E. and Summers, M.D. (1978). Analysis of baculovirus genomes with restriction enzyme endonucleases. *Virology* 98: 517-527.

Smith, G.E. and Summers, M.D. (1979). Restriction maps of five *Autographa californica* MNPV variants, *Trichoplusia ni* MNPV, and *Galleria mellonella* MNPV DNAs with endonuclease *Sma*I, *Kpn*I, *Bam*HI, *Sac*I, *Xho*I, and *Eco*RI. *Journal of Virology* 30: 828-838.

Smith, G.E., Vlak, J.M. and Summers, M.D. (1982). *In vitro* translation of *Autographa californica* nuclear polyhedrosis virus early and late mRNAs. *Journal of Virology* 44: 199-208.

Smith, G.E., Vlak, J.M. and Summers, M.D. (1983a). Physical analysis of *Autographa californica* nuclear polyhedrosis virus transcripts for polyhedrin and 10,000-molecular-weight protein. *Journal of Virology* 45: 215-225.

Smith, G.E., Summers, M.D. and Fraser, M.J. (1983b). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Molecular Cell Biology* 3: 2156-2165.

Summers, M.D. and Arnott, H.J. (1969). Ultrastructural studies on inclusion formation and virus occlusion in nuclear polyhedrosis and granulosis virus-infected *Trichoplusia ni* (Hübner). *Journal of Ultrastructural Research* 28: 462-480.

Summers, M.D. and Smith, G.E. (1987). A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agricultural Experiment Station Bulletin* 1555.

Tanada, Y. and Hess, R.T. (1984). The cytopathology of baculovirus infections in insects. In: *Insect Ultrastructure, Volume 2* (R.C. King and H. Akai, eds), pp. 517-556. Plenum Press, New York.

Vail, P.V., Sutter, G, Jay, D.L. and Gough, D. (1971). Reciprocal infectivity of nuclear polyhedrosis viruses of the cabbage looper and alfalfa looper. *Journal of Invertebrate Pathology* 17: 383-388.

Van der Wilk, F., Van Lent, J.W.M. and Vlak, J.M. (1987). Immunogold detection of polyhedrin, p10 and virion antigens in *Autographa californica* nuclear polyhedrosis virus-infected *Spodoptera frugiperda* cells. *Journal of General Virology* 68: 2615-2623.

Vlak, J.M., Smith, G.E. and Summers, M.D. (1981). Hybridization selection and *in vitro* translation of *Autographa californica* nuclear polyhedrosis virus mRNA. *Journal of Virology* 40: 762-771.

Vlak, J.M. and Rohrmann, G.F. (1985). The nature of polyhedrin. In: *Viral Insecticides for Biological Control*, (K. Maramorosch and K.E. Sherman, eds), pp. 489-544. Academic Press, Orlando, FL.

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klinge-Roode, E.C., Geervliet, J.B.F., Roosien, J. and Van Lent, J.W.M. (1988). Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-ß-galactosidase fusion gene. *Journal of General Virology* 69: 765-776.

Vlak, J.M. and Keus, R.J.A. (1990). Baculovirus expression vector system for production of viral vaccines. In: *Viral Vaccines* (A. Mizrahi, ed). *Advances in Biotechnological Processes* 14: 91-128 . Whiley-Liss, New-York.

Vlak, J.M., Schouten, A., Usmany, M., Belsham, G.J., Klinge-Roode, E.C., Maule, A.J., Van Lent, J.W.M. and Zuidema, D. (1990). Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. *Virology* 179: 312-320.

Vlak, J.M. (1993). Genetic engineering of baculoviruses for insect control. In: *Molecular Approaches to Fundamental and Applied Entomology* (J. Oakeshott and M.J. Whitten, eds), pp. 90-127. Springer-Verlag, New York.

Volkman, L.E. Summers, M.D. and Hsieh, C.H. (1976). Occluded and nonoccluded nuclear polyhedrosis virus grown in *Trichoplusia ni*: Comparative neutralization, comparative infectivity, and *in vitro* growth studies. *Journal of Virology* 19: 820-832.

Volkman, L.E. and Summers, M.D. (1977). *Autographa californica* nuclear polyhedrosis virus: comparative infectivity of the occluded, alkali liberated, and non-occluded forms. *Journal of Invertebrate Pathology* 30: 102-103.

Volkman, L.E. and Zaal, K.J.M. (1990). *Autographa californica* M nuclear polyhedrosis virus: Microtubules and replication. *Virology* 175: 292-302.

Weyer, U. and Possee, R.D. (1988). Functional analysis of the p10 gene 5' leader sequence of the *Autographa californica* nuclear polyhedrosis virus. *Nucleic Acids Research* 16: 3635-3653.

Weyer, U. and Possee, R.D. (1989). Analysis of the promoter of the *Autographa californica* nuclear polyhedrosis virus p10 gene. *Journal of Virology* 70: 203-208.

Whitt, M.A. and 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.

Williams, G.V., Rohel, D.Z., Kuzio, J. and Faulkner, P. (1989). A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *Journal of General Virology* 70: 187-202.

Zanotto, P.M. de A., Kessing, B.D. and Maruniak, J.E. (1993). Phylogenetic interrelationships among baculoviruses: Evolutionary rates and host associations. *Journal of Invertebrate Pathology* 62: 147-164.

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

EXPRESSION OF THE *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS P10 GENE : EFFECT OF POLYHEDRIN GENE EXPRESSION

SUMMARY

Two major late proteins, polyhedrin and p10, are synthesized in large quantities in baculovirus-infected insect cells. This and the fact that both proteins are dispensable for virus replication, form the basis for the use of these viruses as vector for foreign gene expression. To address the question whether the *Autographa californica* nuclear polyhedrosis virus p10 promoter-driven expression is influenced by the concurrent expression of the polyhedrin gene, several recombinants were constructed with various deletions in the polyhedrin gene. The *Escherichia coli* lacZ gene was used as a marker to allow direct comparison between p10 and polyhedrin promoter-driven expression. None of the deletions in the polyhedrin gene did result in higher expression of the p10 promoter-controlled gene. This suggested that the transcriptional and/or translational activity of the p10 and polyhedrin gene are independently regulated. To compare the level of polyhedrin and p10 promoter-driven expression, recombinants with the lacZ gene cloned behind either promoter were studied. No significant difference in level of expression was observed. In cells infected with a recombinant with the lacZ gene present behind both promoters a reduced level of expression was observed, whereas a considerable increase was expected. This may be due to instability of the viral genome, when two copies of the lacZ gene are present.

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INTRODUCTION

Baculoviruses are infectious agents that cause fatal disease in insects. Besides in biocontrol programs of pest insects they have been used extensively to express foreign genes in insect cells (Luckow and Summers, 1988; Luckow, 1991). Two proteins, the polyhedrin protein (29 kDa) and a 10 kDa protein, referred to as p10, are produced in copious amounts in the very late phase (approximately 20-72h p.i.) of infection (Rohel *et al.*, 1983; Smith *et al.*, 1983a). The polyhedrin protein is the major component of the matrix of occlusion bodies, that are formed in the nucleus of baculovirus-infected cells (Rohrmann, 1986). The p10 protein is found associated with fibrillar structures in both nucleus and cytoplasm (Van der Wilk *et al.*, 1987) and may play a role in cell lysis (Williams *et al.*, 1989). Polyhedrin and p10 are not essential for virus production in cultured insect cells (Smith *et al.*, 1983b; Vlak *et al.*, 1988; Williams *et al.*, 1989). Therefore, the promoters of both genes are in principle available to drive high-level expression of foreign genes.

In baculovirus expression vectors the polyhedrin promoter has been routinely used to express foreign genes. Comparison of different polyhedrin-based expression vectors showed, in agreement with promoter studies (Possee and Howard, 1987), that the complete leader sequence of the polyhedrin gene is necessary for high level expression. Higher levels can be obtained when the foreign protein is fused to a few amino-terminal amino acids of polyhedrin (Luckow and Summers, 1988; Matsuura *et al.*, 1987). The p10 promoter has been exploited less often but proved to be useful in the development of vectors expressing more than one foreign gene (Brown *et al.*, 1991) or for production of foreign proteins in insect larvae when an intact polyhedron is important for infection (Vlak *et al.*, 1990). Promoter studies have shown that p10 promoter activity, as was previously found for the polyhedrin promoter, is maximal in the presence of the complete p10 leader sequence (Weyer and Possee, 1988).

The promoter structure of polyhedrin and p10 is very similar. It is AT-rich, contains a conserved 12-nucleotide long consensus sequence, which includes the TAAG motif characteristic for baculovirus late genes (Blissard and Rohrmann, 1990). Transcripts of both genes can be detected in abundance at equivalent levels (Smith *et al.*, 1983a) and polyhedrin ans p10 can accumulate to 50% of the total cell protein content at the end of the infection. These observations raised the question whether simultaneous expression of both the polyhedrin and p10 gene results in limited synthesis of each of the individual proteins. In other words, would it be possible to increase the expression level of one of these genes by deleting the other. Viral or cellular factors involved in polyhedrin and p10 promoter-controlled expression may be limiting to obtain a maximum level of expression. In all expression vectors constructed so far, both promoters are active.

In order to investigate this question various recombinant viruses were analysed in which various deletions in the polyhedrin gene were generated. The p10 coding sequence was replaced by a copy of the lacZ gene to serve as a marker for p10 promoter-driven expression. It is concluded that the polyhedrin and p10 gene are independently regulated.

METHODS

Cell culture and virus

The *Spodoptera frugiperda* cell-line IPLB-SF-21 (Vaughn *et al.*, 1977) was used and maintained in plastic tissue culture flasks in TNM-FH medium (Hink, 1970) supplemented with 10% foetal calf serum. When used for quantitative protein analysis, cells adapted to the protein-free Ex-cell 400™ medium (JR Scientific, Woodland, California) were used. The multiple capsid form of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) strain E2 (Smith and Summers, 1978) was used as wild-type (wt) virus.

Escherichia coli strain DH5α (Gibco-BRL) was used for the bacterial cloning of transfer vectors.

Construction of polyhedrin transfer vectors

All DNA manipulation was performed following the techniques described by Sambrook *et al.* (1989). Restriction enzymes and T4 DNA ligase were from Gibco-BRL; calf intestinal alkaline phosphatase was from Boehringer Mannheim. The plasmid pAcRP23 (Possee and Howard, 1987) was a gift from Dr R.D. Possee (NERC Institute of Virology and Environmental Microbiology, Oxford, UK). Plasmids pAc611 (Luckow and Summers, 1988) and pAc360-ß-gal (Summers and Smith, 1987) were a gift from Dr M.D. Summers (Texas A & M University, College Station, Tx, USA). The structure of the various polyhedrin transfer vectors used, including the derivatives described below, are depicted in Fig. 2.1.

Plasmid pAcJR2 was constructed by replacing the 1.9 kbp *Sph*I-*Bam*HI fragment of pAc611 by the homologous *Sph*I-*Bam*HI fragment from pAcRP23. pAcJR2 contained the complete polyhedrin leader sequence followed by a multiple cloning region with a *Bam*HI, *Sma*I, *Sst*I and *Eco*RI-site. Plasmid pAcJR2 was linearized with *Bam*HI and treated with alkaline phosphatase. A 3.8 kbp *Bgl*II-*Bam*HI fragment derived from plasmid pCH110 (Hall *et al.*, 1983), carrying the lacZ gene, was isolated and inserted in the linearized pAcJR2 giving plasmid pAcES3. The orientation of the insert was checked by restriction enzyme analysis. The lacZ gene used is a chimeric gene with *E. coli* gpt and trpS sequences at the N-terminus to provide the ATG start codon. To obtain plasmid pAcMO7 plasmid pAcJR2 was digested with *Eco*RV and *Sma*I and the 8.5 kbp fragment was isolated and recircularized.

Plasmid pAcMO7 lacks the 0.1 kbp fragment containing the polyhedrin promoter and leader sequences.

Construction of p10 transfer vectors

The 1.9 kbp p10-specific *Eco*RI fragment of AcMNPV was excised from plasmid pAcR159 (Smith *et al.*, 1983a) and ligated to M13mp18 RF DNA that had been linearized with *Eco*RI. Site directed mutagenesis was performed as described by Zoller and Smith (1984) with a primer designed to replace the p10 coding sequence from position +2 to +282, just in front of the TAA stopcodon, by a multilinker containing a *Bam*HI, *Sma*I and *Hin*dIII-site. The resulting 1.6 kbp *Eco*RI-fragment was cloned into pUC4, yielding pAcJJ2. The structure of pAcJJ2 and its derivatives are shown in Fig. 2.2.

Plasmid pAcJJ2 was linearized with *Bam*HI and treated with alkaline phosphatase. Into this linearized vector the same 3.8 kbp *Bgl*II/*Bam*HI fragment carrying the lacZ gene as mentioned above was inserted, resulting in plasmid pAcRK2.

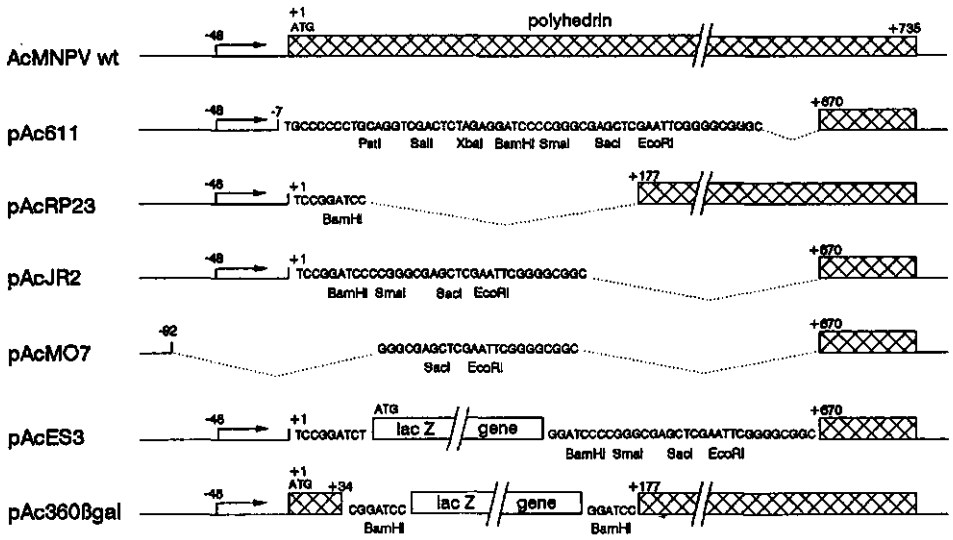

Figure 2.1. Schematic representation of AcMNPV polyhedrin-derived transfer vectors. The structure of the various transfer vectors is given as compared to the wild type (wt) AcMNPV genome segment. The arrows indicate the mRNA start site and the direction of transcription. Position +1 and +735 are the original translational start and stop of polyhdedrin. The hatched area indicate the polyhedrin coding sequence. pAc611 has been described in detail by Luckow and Summers (1988), pAcRP23 by Possee and Howard (1987) and pAc360-ß-gal by Summers and Smith (1987).

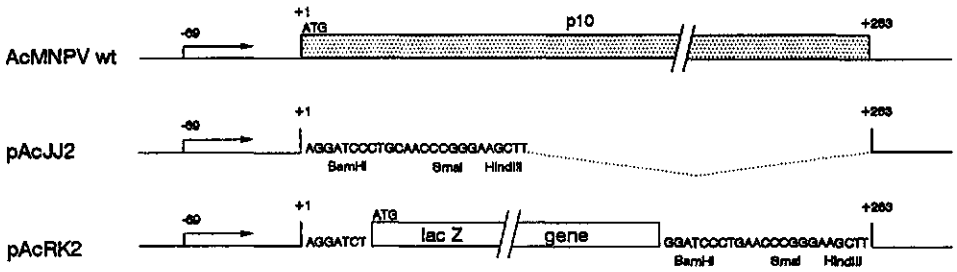

Figure 2.2. Schematic representation of the AcMNPV p10-derived transfer vectors. The DNA sequence of the transfer vectors is aligned with the wild type (wt) AcMNPV genome segment. The arrows indicate the mRNA start site and the direction of transcription. Position +1 and +283 are the original translational start and stop of p10. The dotted area indicates the p10 coding sequence.

Transfection and plaque purification

For transfection experiments viral DNA was extracted from budded virions as was described previously (Vlak *et al.*, 1988). Recombinants AcES3 and AcRK2 were obtained by cotransfecting SF-21 cells with 1 μg wt AcMNPV DNA and 10 μg plasmid DNA (pAcES3 or pAcRK2) using the calcium phosphate precipitation technique essentially as described by Smith *et al.* (1983b) with some minor modifications (Vlak *et al.*, 1988). Double recombinants AcMO1, AcMO2 and AcMO3 resulted from cotransfections of AcRK2 viral DNA with pAcJR2, pAcMO7 or pAcES3 plasmid DNA, respectively.

Recombinant viruses were selected and plaque purified at least four times to obtain genetic homogeneity. Recombinant AcRK2 plaques were selected as blue-colouring plaques upon addition of 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal, Boehringer Mannheim). Recombinant AcES3 and the double recombinants AcMO1, AcMO2 and AcMO3 were selected as blue colouring plaques without polyhedra (Summers and Smith, 1987).

Analysis of recombinants

To analyze recombinant viruses, DNA was extracted from infected cells at 48 h p.i. essentially as described for mammalian cells (Miller *et al.*, 1988). The *Bam*HI restriction pattern was analysed on a 0.7% agarose gel. At the same time p.i. samples were taken for protein analysis according to Laemmli (1970) in a 13.5% SDS-polyacrylamide gel.

B-galactosidase assays

SF-21 cells, adapted to Ex-cell 400™ medium, were seeded at a density of $1x10^6$ cells/ 35 mm Petri dish and allowed to attach for two hours. Cells were inoculated with 20 $TCID_{50}$ units/cell at 27°C with wild type AcMNPV, Ac360-ß-gal, AcES3, AcRK2, AcMO1, AcMO2 and AcMO3 in 750 µl Ex-cell 400™ medium. All incubations were carried out in triplo. After 90 min the inoculum was removed and the cells were washed once with 1 ml medium to minimize the contribution of the ß-galactosidase activity present in the original inoculum. The cells were further incubated for 48 h in 2 ml medium. The cells were sedimented at 1500 rpm for 10 min and were resuspended in cold phosphate-buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, pH 7.0). The supernatants and resuspended pellets were kept on ice. Samples were taken for PAGE and total protein content determinations.

Cells were disrupted by sonication using a sonic disrupter (type B12; Branson Sonic Power Company, Connecticut) for 15 sec at 35 Watt and put on ice. The disruption was checked by light microscopy. The ß-galactosidase activity was measured using the ONPG-test as described by Miller (1972). Both supernantant and sonicated cells were measured at a dilution which gave a good measurable yellow colour within 10 minutes of reaction at 37°C. From the absorbance values at 420 nm the ß-galactosidase activity/ml was calculated using the formula:

$$U = \frac{A_{420} \cdot V}{\varepsilon . d . T . v} . D$$

where U is the number of ß-galactosidase units per ml sample. One unit of enzyme activity is defined as the amount of enzyme liberating 1 µmol ONP per minute at 37°C; V is the total volume at the end of the reaction, ϵ the extinction coefficient of ONP at pH 11 (3.5 $\mu mol^{-1}.ml.cm^{-1}$), d the optical way length, T the reaction time, v the added sample volume, and D the dilution factor of the sample. The activity per 10^6 cells was calculated from the measured activities of both sonicated cell suspension and medium fractions.

The proteins were analysed by electrophoresis in a 13.5% SDS-polyacrylamide gel (Laemmli, 1970). The amount of protein present in cells infected with the various viruses was determined with the BioRad protein assay system following the manufacturer's protocol. This system exploits the shift in absorbance at 595 nm of Coomassie brilliant blue upon complexion with proteins (Bradford, 1976).

RESULTS

Construction of AcMNPV recombinants

In order to study the effect of polyhedrin gene deletions on the expression of p10 promoter-driven genes several lacZ-expressing recombinants were made (Fig. 2.3.). Recombinants AcES3 and AcRK2 resulted from homologous recombination between wild type AcMNPV DNA and the transfer vectors pAcES3 and AcRK2, respectively. AcES3 contained the lacZ gene under polyhedrin promoter control and had a wild type p10 gene. AcRK2 contained the lacZ gene under p10 promoter control and had a wild type polyhedrin gene. Recombinants AcMO1, AcMO2 and AcMO3 were obtained by recombination between AcRK2 viral DNA and pAcJR2, pAcMO7 and pAcES3 plasmid DNA, respectively.

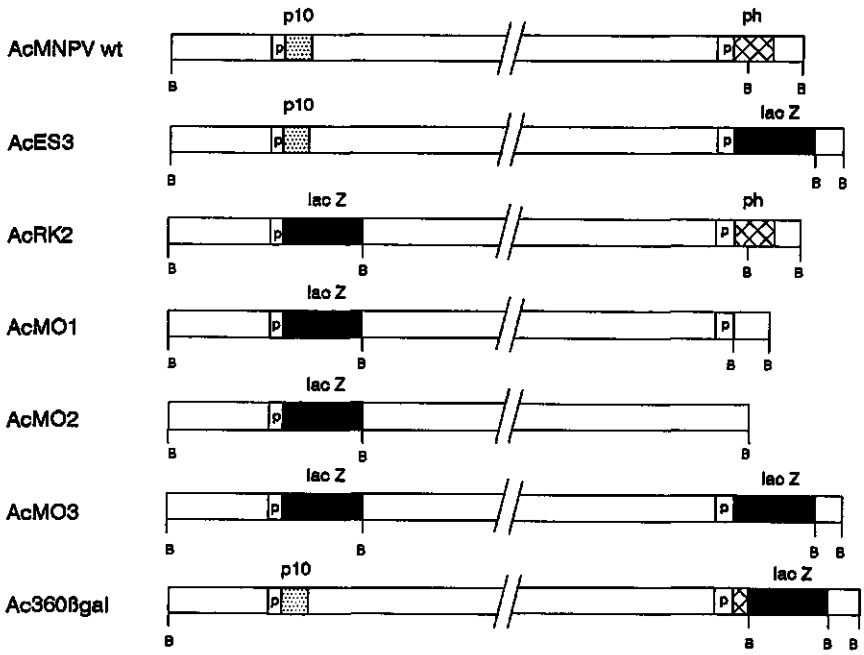

Figure 2.3. Structure of the adjacent *Bam*HI-B (23 kbp) and F (1.9 kbp) segments of wild type (wt) AcMNPV extending from map position 85.0 to 4.8 (Vlak and Summers, 1982) and containing the p10 and polyhedrin gene. The corresponding segments of the recombinant virus genomes are aligned. *Bam*HI sites are indicated as B. In the p10 recombinants an additional *Bam*HI site is introduced in the original *Bam*HI-B segment as a cloning site. Hatched areas indicate polyhedrin coding sequences, grey dotted areas indicate p10 coding sequences and darkly grey dotted areas indicate lacZ sequences.

These double-recombinants all expressed lacZ under p10 promoter control but varied in the polyhedrin gene. AcMO1 contained only the polyhedrin promoter and 5' leader sequences, but lacked the amino acid coding sequence (+1 to +670) of polyhedrin including the ATG translational start. In AcMO2 the entire polyhedrin gene was deleted including the complete promoter region. AcMO3 contained lacZ under the control of both promoters. The identity of the recombinant viruses was verified by restriction enzyme analysis of viral DNA (data not shown).

SDS-PAGE analysis of recombinant AcMNPV-infected cells

Cells were grown in protein-free medium and infected with the various recombinant viruses. Protein-free medium was chosen to eliminate washing steps to remove bovine serum albumin before SDS-PAGE, thus avoiding loss of ß-galactosidase activity. Uninfected cells, and cells infected with wt virus or with recombinant Ac360-ß-gal were used as controls. The infected cells were harvested 48 h p.i. and the proteins analysed by SDS-PAGE. Polyhedrin and p10 were expressed as expected and cells infected with all recombinants showed expression of ß-galactosidase (Fig. 2.4). The polyhedrin recombinant AcES3 (Fig. 2.4, lane 4) and the p10 recombinant AcRK2 (Fig. 2.4, lane 5) produced equal amounts of ß-galactosidase, suggesting that the polyhedrin and p10 promoters are equally strong. However, for AcES3 the level of expression varied and was sometimes higher than for AcRK2.

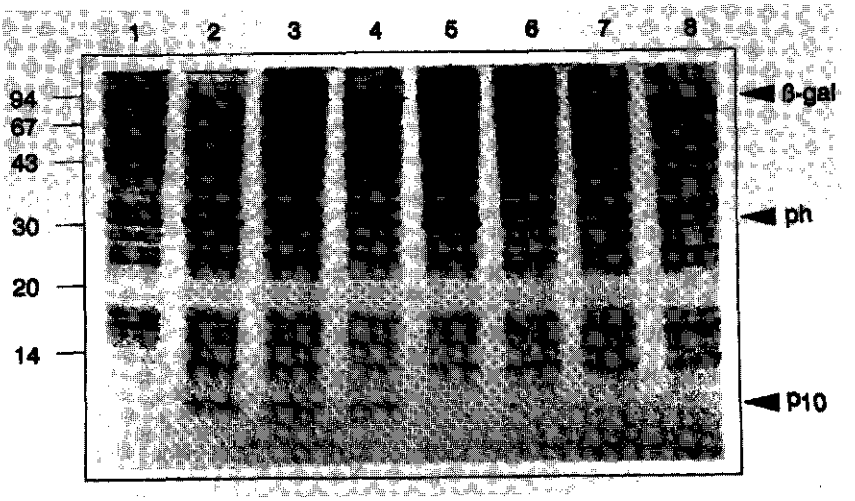

Figure 2.4. *Spodoptera frugiperda* cells (1) infected with wt AcMNPV (2) and the various recombinants Ac360-ß-gal (3), AcES3 (4), AcRK2 (5), AcMO1 (6), AcMO2 (7) and AcMO3 (8) were harvested 48 h post infection and analysed in a 13.5 % SDS-polyacrylamide gel. Each lane contains the equivalent of 4.10^4 cells. Molecular weight markers are indicated in 10^3 Da.

Similar amounts of ß-galactosidase were also produced in cells infected with recombinants AcMO1 and AcMO2, containing deletions in the polyhedrin gene (Fig. 2.4, lanes 6 and 7). Cells infected with recombinant AcMO3, which contained two copies of the lacZ gene, produced considerably less ß-galactosidase (Fig. 2.4, lane 8). The supernatants of infected cells also contained some ß-galactosidase as judged from SDS-PAGE (data not shown), probably due to lysis of cells or active secretion.

Table 2.1. Levels of ß-galactosidase activity measured at 48 h p.i. in cells and medium infected with the various recombinant viruses[1].

	ß-galactosidase activity (U/10^6 cells) (± SD)		
Virus	cells	medium	total
Mock	-	-	-
AcMNPV (wt)	-	-	-
Ac360-ß-gal	39 (± 5.5)	13 (± 0.5)	54 (± 6.8)
AcES3	23 (± 7.3)	6.7 (± 1.2)	30 (± 8.3)
AcRK2	19 (± 2.6)	4.1 (± 0.6)	23 (± 2.6)
AcMO1	13 (± 0.8)	3.4 (± 0.4)	17 (± 0.8)
AcMO2	19 (± 0.8)	3.5 (± 0.3)	23 (± 1.1)
AcMO3	8.6 (± 1.2)	2.0 (± 0.2)	11 (± 1.1)

[1] Infections were carried out in triplicate. All individual measurements were done in duplicate to confirm the accuracy of the ONPG assay. The variation between the different infections is expressed by the standard deviation (within brackets).

Enzymatic analysis of recombinant AcMNPV-infected cells

A quantitative analysis of the production of ß-galactosidase in cells infected with the various recombinants was performed. Cells and supernatants were harvested at 48 h p.i. and analysed using ONPG as substrate to test the enzyme activity (Table 2.1). From the absorption values at 420 nm the ß-galactosidase activity in cells and supernantants was calculated per 10^6 cells (Table 2.1). The results of these determinations confirmed the observations from the PAGE (Fig. 2.4) in that deletions in the polyhedrin gene did not increase expression of ß-galactosidase driven by the p10-promoter. In fact, the ß-galactosidase expression by AcMO1, in which only the coding sequence of the polyhedrin gene was deleted, was slightly lower than the expression by AcMO2, which lacked the entire polyhedrin gene. The fusion mutant

Ac360-ß-gal expressed more ß-galactosidase than all other recombinants. This is in agreement with similar findings of Matsuura *et al.* (1987) and of Luckow and Summers (1988), that fusion recombinants produce more product than non-fusions. The recombinant expressing ß-galactosidase under both promoters (AcMO3) showed reduced levels of the enzyme. The same overall results were obtained when the enzyme activities were calculated per μg protein (data not shown).

DISCUSSION

The hyperactivity of the baculovirus polyhedrin and p10 promoters has been widely exploited to express foreign genes (Luckow and Summers, 1988; Luckow, 1991). Since both genes have a comparable promoter structure (Rohrmann, 1986) and are expressed concurrently during the late stages of infection, competition might occur for transcriptional or translational factors, thereby reducing the potential level of expression of each of the proteins. To investigate this, recombinants were constructed lacking either the coding sequence (AcMO1) or the complete polyhedrin gene including the promoter (AcMO2). The effect on p10 promoter-driven gene expression was measured and, to facilitate quantification, a foreign protein with enzymatic activity (ß-galactosidase) was chosen as a marker for p10 expression (AcRK2).

The ß-galactosidase expression levels were comparable for AcMO2 and AcRK2, indicating that the activity of the p10 gene is not affected by the presence of a functional polyhedrin gene (Fig. 2.4, Table 2.1). This suggests that the p10 and polyhedrin genes are regulated by independent mechanisms. The results also indicate that the maximum level of p10 expression is not determined by limitations in the availability of cellular or viral factors, which are required for transcription and/or translation of both the p10 and polyhedrin gene. When the polyhedrin coding sequence was removed, while the polyhedrin promoter was maintained (AcMO1), the expression from the p10 promoter was even at a lower level than when the complete polyhedin promoter was present (AcRK2). It might be that, in the case of AcMO1, the polyhedrin promoter became hyper-active, thereby removing transcriptional factors, which are essential for both polyhedrin and p10 gene expression. In turn, this may imply that wild-type polyhedrin gene expression is regulated by a feed back mechanism. Transcript analysis should reveal whether the polyhedrin promoter becomes hyperactive or not in AcMO1-infected cells.

The double lacZ recombinant AcMO3 showed reduced ß-galactosidase production as compared to the single lacZ recombinants AcRK2 and AcES3, whereas a considerable increase was expected. It was observed that plaque purification of this recombinant was cumbersome and that it was difficult to obtain high titre stocks. Since the polyhedrin and the

p10 gene are orientated in the same direction in the viral genome (Kuzio *et al.*, 1984; Smith *et al.*, 1983a), the lacZ sequences were also orientated in the same direction. Hence, the most plausible explanation for the low expression of the AcMO3 recombinant may be internal recombination, leading to lower numbers of viral genome copies per cell. The use of a lacZ gene with a different nucleotide sequence may alleviate this problem.

There is further evidence to suggest, that the polyhedrin and p10 genes are independently regulated. The onset of p10 gene expression precedes polyhedrin gene expression by about 4 hours (Roelvink *et al.*, 1992), indicating that these genes have a different pattern of temporal expression. It is also possible that the expression of polyhedrin and p10 or ß-galactosidase is spatially separated in the cells. Since doubling of the polyhedrin promoter (Emery and Bishop, 1987) to simultaneously drive the expression of polyhedrin protein and the LCMV-N protein resulted in high level expression of both proteins, the level of polyhedrin expression itself does not seem to be controlled by limited viral or cellular transcriptional factors. Likewise, duplication of the p10 promoter was not shown to have any influence on the levels of polyhedrin and p10 production (Weyer *et al.*, 1990). The number of polymerases that can initiate transcription on both the polyhedrin and p10 promoter may be restricted by the rate and efficiency of initiation and elongation.

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REFERENCES

Blissard, G.W. and Rohrmann, G.F. (1990). Baculovirus diversity and molecular biology. *Annual Review of Entomology* 35: 127-155.

Bradford, M. (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.

Brown, C.S., Van Lent, J.W.M., Vlak, J.M. and Spaan, W.J.M. (1991). Assembly of empty capsids by using baculovirus recombinants expressing human parvovirus B19 structural proteins. *Journal of Virology* 65: 2702-2706.

Emery, V.C. and Bishop, D.H.L. (1987). The development of multiple expression vectors for high level synthesis of eukaryotic proteins: expression of LCMV-N and AcNPV polyhedrin protein by a recombinant baculovirus. *Protein Engineering* 1: 359-366.

Hall, C.V., Jacob, P.E., Ringold, G.M. and Lee, F. (1983). Expression and regulation of *Escherichia coli* lacZ gene fusions in mammalian cells. *Journal of Molecular and Applied Genetics* 2: 101-109.

Hink, F. (1970). Established insect cell line from the cabbage looper, *Trichoplusia ni*. *Nature (London)* 226: 446-467.

Kuzio, J., Rohel, D.Z., Curry, C.J., Krebs, A., Carstens, E.H. and Faulkner, P. (1984). Nucleotide sequence of the p10 polypeptide gene of *Autographa californica* nuclear polyhedrosis virus. *Virology* 139: 414-418.

Laemmli, UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227: 680-685.

Luckow, V.A. and Summers, M.D. (1988). Signals important for high-level expression of foreign genes in *Autographa californica* nuclear polyhedrosis virus expression vectors. *Virology* 167: 56-71.

Luckow, V.A. (1991). Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In: *Recombinant DNA Technology and Applications* (A. Prokop, R.K. Bajhap and C.S. Ho, eds), pp. 97-152. McGraw-Hill, Inc., New York.

Matsuura, Y., Possee, R.D., Overton, H.A. and Bishop, D.H.L. (1987). Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. *Journal of General Virology* 68: 1233-1250.

Miller, J.H. (1972). *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* 16: 3.

Possee, R.D. and Howard, S.C. (1987). Analysis of the polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus. *Nucleic Acids Research* 15: 10233-10248.

Rohel, D.Z., Cochran, M.A. and Faulkner, P. (1983). Characterization of two abundant mRNAs of *Autographa californica* nuclear polyhedrosis virus present late in infection. *Virology* 124: 357-365.

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

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

Smith, G.E. and Summers, M.D. (1978). Analysis of baculovirus genomes with restriction enzyme endonucleases. *Virology* 89: 517-527.

Smith, G.E., Vlak, J.M. and Summers, M.D. (1983a). Physical analysis of *Autographa californica* nuclear polyhedrosis virus transcripts for polyhedrin and 10,000-molecular-weight protein. *Journal of Virology* 45: 215-225.

Smith, G.E., Fraser, M.J. and Summers, M.D. (1983b). Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: deletion mutations within the polyhedrin gene. *Journal of Virology* 46: 584-593.

Summers, M.D. and Smith, G.E. (1987). A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agricultural Experiment Station Bulletin* 1555.

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

Vlak, J.M. and Summers, M.D. (1982). Orientation of the genome of *Autographa californica* nuclear polyhedrosis virus: a proposal. *Journal of Virology* 41: 1119-1121.

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klinge-Roode, E.C., Geervliet, J.B.F., Roosien, J. and Van Lent, J.W.M. (1988). Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-ß-galactosidase fusion gene. *Journal of General Virology* 69: 765-776.

Vlak, J.M., Schouten, A., Usmany, M., Belsham, G.J., Klinge-Roode, E.C., Maule, A.J., Van Lent, J.W.M and Zuidema, D. (1990). Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. *Virology* 179: 312-320.

Weyer, U. and Possee, R.D. (1988). Functional analysis of the p10 gene 5' leader sequence of the *Autographa californica nuclear* polyhedrosis virus. *Nucleic Acids Research* 16: 3635-3653

Weyer, U., Knight, S. and Possee, R.D. (1990). Analysis of very late gene expression by *Autographa californica* nuclear polyhedrosis virus and the further development of multiple expression vectors. *Journal of General Virology* 71: 1525-1534.

Van der Wilk, F., Van Lent, J.W.M. and Vlak, J.M. (1987). Immunogold detection of polyhedrin, p10 and virion antigens in *Autographa californica* nuclear polyhedrosis virus-infected *S. frugiperda* cells. *Journal of General Virology* 68: 2615-2623.

Williams, G.V., Rohel, D.Z., Kuzio, J. and Faulkner, P. (1989.) A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *Journal of General Virology* 70: 187-202.

Zoller, M.J. and Smith, M. (1984). Oligonucleotide-directed mutagenesis: A simple method using two oligonucleotide primers and a single-strand DNA template. *DNA* 3: 479-488.

NUCLEOTIDE SEQUENCE OF THE P10 GENE OF *SPODOPTERA EXIGUA* NUCLEAR POLYHEDROSIS VIRUS

SUMMARY

The p10 gene of *Spodoptera exigua* multiple nuclear polyhedrosis virus (SeMNPV) was localized on the *Xba*I fragment H (5.1 kbp) of the physical map of the viral genome. The coding sequence of the SeMNPV p10 gene is 264 nucleotides (nt) long, corresponding to a predicted protein of 88 amino acids with a molecular weight of 9,607 Da. The SeMNPV p10 protein showed only limited amino acid identity (39% and 26%, respectively) to the p10 proteins of *Orgyia pseudotsugata* MNPV (OpMNPV) and *Autographa californica* MNPV (AcMNPV) and thus appears less conserved than other viral proteins. The transcript of the p10 gene could start in the conserved baculovirus late gene promoter motif TAAG. The SeMNPV p10 promoter is AT-rich (92%) and has the shortest leader of all baculovirus major late genes reported so far. A putative poly(A) signal (AATAAA) is located 61 nt downstream of the translational stop codon TAA. Partial putative ORFs were found up- and downstream of the p10 gene with significant homology to the baculovirus p26 and p74 proteins. It is concluded that the region of SeMNPV DNA containing the p10 gene is collinear with the corresponding regions in the AcMNPV and OpMNPV genomes.

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INTRODUCTION

Nuclear polyhedrosis viruses (NPVs), classified in the family *Baculoviridae* (Francki *et al.*, 1991), produce two polypeptides, polyhedrin and p10, at very high levels in the late phase of infection. At 48 h post-infection (p.i.) of *Spodoptera frugiperda* cells with *Autographa californica* nuclear polyhedrosis virus (AcMNPV) these two polypeptides constitute about half of the protein mass in insect cells (Smith *et al.* 1982). Polyhedrin is the major constituent of the viral occlusion bodies, which are found exclusively in the nuclei of baculovirus-infected insect cells (Vlak and Rohrmann, 1985). The p10 protein is associated with large fibrillar structures in both nuclei and cytoplasm of infected cells (Summers and Arnott, 1969; Van der Wilk *et al.*, 1987; Williams *et al.*, 1989; Russell *et al.*, 1991). In view of its abundance and the particular structures formed in infected cells, it is surprising that no further information is available on the functions of the p10 protein. Studies with p10 mutants of AcMNPV have shown that the p10 protein is not essential for viral replication either *in vivo* or *in vitro*, but cells infected with these p10 mutants failed to liberate polyhedra (Vlak *et al.*, 1988; Williams *et al.*, 1989).

Only two baculoviral p10 genes have been studied in detail. The p10 coding sequences of AcMNPV (Kuzio *et al.*, 1984) and *Orgyia pseudotsugata* MNPV (OpMNPV) (Leisy *et al.*, 1986a) are similar in size, 282 and 276 nucleotides (nt), respectively. In contrast to the highly conserved polyhedrin genes with 80% nucleotide sequence and 90% amino acid sequence identity (Leisy *et al.*, 1986a), the p10 genes have only 54% and 41% identity at the nucleotide and amino acid sequence levels, respectively, with the highest homology in the amino terminus (Leisy *et al.*, 1986b). Hence, despite the high degree of homology between the AcMNPV and OpMNPV genomes (Rohrmann, 1992) the p10 genes have diverged considerably. It is therefore of interest to investigate the p10 genes of more distantly related baculoviruses and to determine to what extent the structural homology in the p10 protein is preserved. *Spodoptera exigua* MNPV (SeMNPV) is a baculovirus specific for a single insect species, the beet army worm *Spodoptera exigua*, whereas AcMNPV for example can infect over 30 insect species. Based upon the comparison of polyhedrin genes SeMNPV appeared distantly related to AcMNPV and OpMNPV (Van Strien *et al.*, 1992). This chapter describes the identification and subsequent sequence analysis of the SeMNPV p10 gene.

MATERIALS AND METHODS

Cells and virus

The *Spodoptera exigua* cell line UCR-SE-1 (Gelernter and Federici, 1986b), obtained from Dr B.A. Federici (Department of Entomology, University of California, Riverside, Ca., U.S.A.) was used and maintained in plastic tissue culture flasks (NUNC) in TNM-FH medium (Hink, 1970) supplemented with 10% fetal calf serum. A field isolate of SeMNPV (Gelernter and Federici, 1986a), kindly provided by Dr B.A. Federici, was used to infect fourth instar larvae of *S. exigua* (Smits *et al.*, 1988). The haemolymph of these infected larvae was used to obtain budded virus for infection of cultured UCR-SE-1 cells.

RNA isolation and cDNA synthesis

Total RNA was isolated from SeMNPV-infected cells at 24, 48 and 74 h post infection (p.i.) essentially as described by Van Strien *et al.*, 1992. Polyadenylated RNA was selected from the 74 h p.i. sample by oligo(dT)-cellulose column chromatography (Sambrook *et al.*, 1989) and served as template in a reverse transcription reaction. Poly(A)$^+$ RNA (1.5 μg) and oligo(dT)$_{12\text{-}18}$ (500 ng) were incubated in a volume of 5 μl for 5 min at 70°C and for 1 h at 42°C in a reaction volume of 50 μl after addition of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT, 20 μmCi (α-^{32}P) dATP, 20 μCi (α-^{32}P) dCTP, 1mM dGTP, 1 mM dTTP, 80 units RNasin, 4 mM sodium pyrophosphate and 28 units of reverse transcriptase (Life-Technologies). After alkali degradation of the template RNA, cDNA was purified over a Sephadex G50 column and directly used in Southern blot hybridization.

Viral DNA isolation, Southern blot hybridization and cloning

SeMNPV DNA was isolated from virions, which were liberated from polyhedra upon alkali treatment and cleared by sucrose gradient centrifugation (Caballero *et al.*, 1992). SeMNPV DNA was digested with various restriction enzymes, electrophoretically separated in 0.7% agarose gels using standard techniques (Sambrook *et al.*, 1989) and transferred under alkaline conditions to Hybond-N nylon membranes (Amersham) by capillary transfer, according to the manufacturer's specifications. After prehybridization at 60°C for 3 h in 6 x SSC, 0.5% (w/v) SDS, 1% Ficoll-400, 1% polyvinylpyrrolidone, 1% BSA (fraction V) and 100 μg/ml herring sperm DNA, blots were hybridized at 60°C for 20 h to a ^{32}P-labelled cDNA probe (1 x 10^6 c.p.m./ml) synthesized from poly(A)$^+$ RNA isolated from SeMNPV-infected cells 74 h p.i. (see above). After hybridization membranes were washed twice for 5 min at room temperature in 4 x SSC, 0.1% (w/v) SDS, and twice for 10 min in 2 x SSC, 0.1% SDS at 45°C. Membranes were exposed to Kodak XAR-5 X-ray film using intensifying screens.

Based on the hybridization with the cDNA probe, *Xba*I fragment H of SeMNPV DNA and accompanying subfragments were isolated from agarose gels by the freeze-squeeze method (Tautz and Renz, 1983) and cloned in plasmid pTZ18R or pBluescript SK^+.

DNA sequencing and computer analysis

All sequencing reactions were performed on double-stranded DNA plasmid templates using T7 DNA polymerase and the chain termination method of Sanger *et al.* (1977). Sequence data were analysed with the UWGCG computer programs (Devereux *et al*, 1984). The DNA sequence and the deduced amino acid sequences of open reading frames (ORFs) were compared with sequences in the updated Genbank/EMBL, SWISS PROT and NBRF databases, using the FASTA and TFASTA programs.

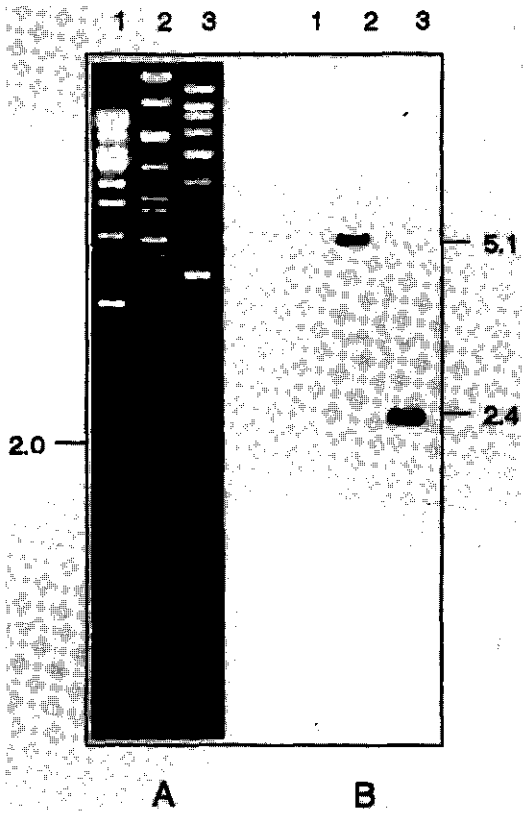

Figure 3.1. Localization of the SeMNPV p10 gene. A) Restriction enzyme analyses of AcMNPV DNA digested with *Eco*RI (lane 1) and SeMNPV DNA digested with *Xba*I and *Sph*I (lanes 2 and 3. respectively). B) Southern blot hybridization of (A) with cDNA as probe, which was obtained by reverse transcription of poly(A)$^+$ RNA extracted from SeMNPV-infected insect cells 74 h p.i. The sizes of the AcMNPV fragment *Eco*RI-P (2.0 kbp), and the SeMNPV fragments *Xba*I-H (5.1 kbp) and *Sph*I-R (2.4 kbp) are indicated.

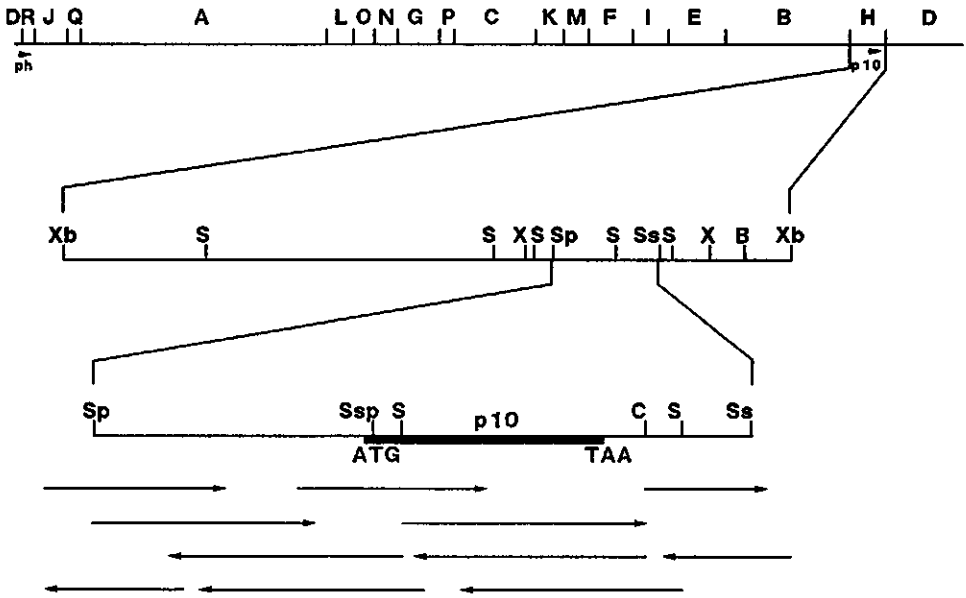

Figure 3.2. Location of the p10 gene on the physical map for *Xba*I restriction endonuclease of the SeMNPV genome and sequence strategy for the p10 gene region. The *Sph*I-*Sst*I region (914 bp) of *Xba*I-H was sequenced. The location of the SeMNPV p10 gene is indicated (thick line). The arrows below the lower map indicate the sequencing strategy of the 914 bp region. The location of the previously identified polyhedrin gene (ph) is shown as a reference (Van Strien *et al.*, 1992). B = *Bam*HI; C = *Cla*I; S = *Sal*I;, Sp = *Sph*I;, Ss = *Sst*I;, Ssp = *Ssp*I; X = *Xho*I; Xb = *Xba*I.

RESULTS

Mapping, cloning and sequencing of the SeMNPV p10 gene

Southern blot hybridization at low stringency, using the AcMNPV p10 gene as a heterologous probe (a 555 bp *Xho*I-*Ssp*I fragment of *Eco*RI fragment P of AcMNPV DNA), did not result in the detection of a similar sequence on the SeMNPV genome. Assuming that SeMNPV p10 mRNA occurs abundantly late after infection (Smith *et al.*, 1983), poly(A)$^+$ RNA was isolated from mock- and SeMNPV-infected cells 74 h p.i. and used as template for cDNA synthesis. This radio-labelled cDNA was then hybridized to Southern blots of SeMNPV DNA digested with *Xba*I and *Sph*I (Fig. 3.1 A, lane 2 and 3, respectively). Strong hybridization signals were observed to an *Xba*I fragment of 5.1 kbp (*Xba*I-H) and an *Sph*I fragment of 2.4 kbp (*Sph*I-R) was observed (Fig. 3.1 B, lanes 2 and 3). Since SeMNPV polyhedrin

```
SphI                                                          -320
GCATGCGGCC CGCGGGTTTA ATTTCTGGTC AGATGATGTT CGACGACCGC GTGATCGTCA
   M  R  P   A  G  L   I  S  G   Q  M  M  F   D  D  R   V  I  V

                                                              -260
AAAAGCTAAA GGCCGACATG GCGGTGTACG GTCGTCAACA GTTGCCGTAT AGCAGTGCCC
K  K  L  K   A  D  M   A  V  Y   G  R  Q  Q   L  P  Y   S  S  A

                                                              -200
ACATGTCCGC GAAACAATTT GCAATGGCGG CGACGGTAAA CAGGCAATTG TACCGCGACC
H  M  S  A   K  Q  F   A  M  A   A  T  V  N   R  Q  L   Y  R  D

                                                              -140
TGCCACGATA CGCGGTCGTG TTCCACAACA ACACCGACAT TACCATCACG ATGGTCGAGG
L  P  R  Y   A  V  V   F  H  N   N  T  D  I   T  I  T   M  V  E

                                                               -60
GCGAGTTTGA AATGTATCGA GTCCGATTGG ACGGACCTCT GATTACGAAT CAGAACAAAG
G  E  F  E   M  Y  R   V  R  L   D  G  P  L   I  T  N   Q  N  K

                                                poly(A)signal -20
ACGACGACGA CGACGACAGT ATTGGCAATA TCGTATAGAG AATAAGTTTA TTATTATAAT
D  D  D  D   D  D  S   I  G  N   I  V  *          ^^^^ ^^

                    +1                                         41
TGTAATTATA TTATACATTA TGAGTCAAAA TATTTTACTT TTGATCCGAG CCGACATTAA
                    M  S  Q  N   I  L  L   L  I  R   A  D  I  K

                                                              101
AGCTGTCGAC GAAAAAGTCG ATGCTTTGCA GCAGGCCGTC AACGACGTGT CTGCCAATTT
  A  V  D   E  K  V   D  A  L  Q   Q  A  V   N  D  V   S  A  N  L

                                                              161
GCCCGATACT TCAGAGCTGT CGGCCAAATT AGACGCTCAG GCCACCACCC TAGACACCAT
  P  D  T   S  E  L   S  A  K  L   D  A  Q   A  T  T   L  D  T  I

                                                              221
TGTCACCCAA GTGAACAACA TCAACGACGT GCTCAATCCC GATCTGCCCG ACGTGCCTGG
  V  T  Q   V  N  N   I  N  D  V   L  N  P   D  L  P   D  V  P  G

                                                              281
CAATCTACAA AAGCAGCAAC AGCAAAAGAA AAGCAACAAA AAGTAAAACT TAACGACGAC
  N  L  Q   K  Q  Q   Q  Q  K  K   S  N  K   K  *

                                                poly(A)signal 341
GACGACGACG ACGATTGTGT ATTTGAGCAT GTTTGTAGTA TTATAAAATA AAAAATTTTG
                                                   ^^^^ ^^

                                                              401
GCCAAATTTC GTTGTGTATT ATTCCGAATA GAGATTGTCG TACCATCGAT TCTGTAAATA
                    *   E  S  Y   L  N  D   Y  W  R  N   Q  L  Y

                                                              461
GTTGGTTTGT CGTCGTAAAT TGACGTAGTA TGATAGTGCG TCGACAAGGA GGGTGTACAG
 N  T  Q   R  R  L  N   V  Y  Y   S  L  A   D  V  L  L   T  Y  L

                    poly(A)signal                             521
GGCTAAAATT AAAAATATAA TAAAGAGCGC CGTCACGTTC GTGTCGCGGG GCATCATCGC
 A  L  I   L  F  I  I   F  L  A   T  V  N   T  D  R  P   M  M  A
                      ^^ ^^^^

        SstI
GAGAACGAGA GCTC
 L  V  L   A
```

Figure 3.3. Nucleotide sequence of a 914 bp region of the SeMNPV genome containing the p10 gene. The p10 coding sequence starts at postion +1 and terminates at position +267. The predicted amino acid sequence is indicated by the one-letter code. The transcription initiation signal (TAAG) is underlined. Putative transcription termination signals (AATAAA) are indicated by arrow heads.

mRNA was not polyadenylated (Van Strien *et al.*, 1992), this hybridization signal was most likely due to cDNA derived from p10 transcripts.

*Xba*I fragment H was cloned and a detailed physical map for various restriction enzymes was constructed (Fig. 3.2). After hybridization with radio-labelled cDNA as described above, subclones were identified and a 914 bp *Sph*I-*Sst*I fragment was sequenced (Fig. 3.3). It contained an ORF of 264 nt with the potential to encode a protein of 88 amino acids with a predicted molecular weight of 9,607 Da. This ORF presumably represented the p10 gene of SeMNPV (see below).

The 5' non-coding sequence of the SeMNPV p10 gene contained a TAAG motif, the consensus sequence for the transcriptional start site of baculovirus late and very late genes (Blissard and Rohrmann, 1990). The TAAG consensus sequence was located at position -37 to -34 with respect to the translational start codon of the SeMNPV p10 ORF (Fig. 3.3). A putative conserved motif ATTGTA, suggested to be involved in ribosome binding (Zanotto *et al.*, 1992), was identified two DNA helix turns downstream of the putative late transcription start site TAAG of the SeMNPV p10 gene. A sequence motif consisting of six repeats of the triplet GAC was found upstream (position -63 to -80) and downstream (positions +276 to +293) of the p10 polypeptide coding sequence. Two putative poly(A) signal sequences (AATAAA) were found in the 3' non-coding region of the putative p10 gene, located approximately 60 nt and 210 nt downstream of the translational stop codon.

Contiguous ORFs were identified upstream and downstream of the p10 gene, and these extended beyond the sequenced region (Fig. 3.3). The p10 gene and the upstream ORF had the same polarity, whereas the downstream ORF had an opposite polarity. The partial identified upstream ORF had a high amino acid sequence similarity (53% and 55%, respectively) to the p26 genes of AcMNPV and OpMNPV, while the determined sequences of the downstream ORF had a high amino acid similarity (77% and 71%, respectively) to the p74 genes of these viruses. These partial ORFs thus were better conserved than the p10 gene. Based on these data it is likely that the 264 nt ORF indeed encodes the SeMNPV p10 protein, assuming that the genetic organization of SeMNPV in the p10 containing region is collinear with the p10 gene and flanking regions of AcMNPV and OpMNPV.

Comparison of the p10 genes of SeMNPV, AcMNPV and OpMNPV

The SeMNPV p10 ORF (264 nt) was similar in size compared to the p10 genes of OpMNPV (276 nt) and AcMNPV (282 nt). The primary sequence of these genes has diverged considerably in these three viruses. The overall amino acid sequence identity was 26 to 39% (Table 3.1 and Fig. 3.4). Despite this sequence divergence the hydrophilicity plots showed a reasonable similarity between the three p10 proteins (Fig. 3.5). A small hydrophobic region was present near the amino terminus of the protein, whereas the carboxy terminus was highly

hydrophilic and contained several basic amino acid residues. Furthermore, the carboxy-terminal half of all three p10 proteins shared a high proline content. The p10 proteins did not contain cysteine, tryptophan or histidine residues. Dotplot comparison between the three different p10 proteins showed two stretches of homology common to all three proteins (Fig. 3.6). The position of the first homology box KV(D/T)(A/S)(L/V)Q was conserved in the primary structure of the p10 proteins (Fig. 3.4), but the second conserved homology box (L/V/I)PD(V/L)P was located at different positions in the primary structure.

The leaders of the three p10 genes differed considerably in size (approximately 36 nt in SeMNPV, 52 nt in OpMNPV and 70 nt in AcMNPV), but were all AT rich (92%, 77% and 87%, respectively). The trailer sequences of the p10 genes (approximately 85 nt in SeMNPV, 175 nt in OpMNVP, and 162 nt in AcMNPV) had no additional features in common, other than that they all contained the eukaryotic consensus poly(A) signal sequence AATAAA (Birnstiel *et al.*, 1985).

```
SeMNPV   M S Q - N I L L L I R A D I K A V D E K V D A L Q Q A V N D V    30
OpMNPV   . . K P S . . T Q . L D A V R . . . S . . T . . . T Q . D Q L    31
AcMNPV   . . K P N V . T Q . L D A V T E T N T . . D S V . T Q L N G L    31
                                                 * * * * * *

SeMNPV   S A N L P D T S E L S A K L D A Q A T T L D T I V T Q V N N I    61
OpMNPV   V E D S K T L E A . T D Q . G E L D N K V S D . Q S M L S V E    62
AcMNPV   E E S F Q L L D G . P A Q . T D L N T K I S E . Q S I L T G D    62

SeMNPV   N D V L N P D L P D V P G N L Q K Q Q Q Q K K S N K K            88
OpMNPV   E E L P E . P A . A P E P E . P E I P D V P G L R R S R K Q      92
AcMNPV   I V P D L . D S L K P K L K S Q A F E L D S D A R R G K R S S K  94
```

Figure 3.4. Alignment of the deduced amino acid sequence of the p10 proteins of SeMNPV, OpMNPV (Leisy *et al.*, 1986a) and AcMNPV (Kuzio *et al.*, 1984). Homology box KV/(D/T)(A/S)(L/V)Q is indicated by asterisks and homology box (L/I/V)PD(V/L)P has been underlined. Identical amino acids are indicated by dots. The hyphen represents a gap introduced to maximize matching of amino acid residues. Numbers indicate amino acid positions.

Table 3.1. Nucleotide sequence identity and amino acid sequence identity or similarity between the p10 genes of SeMNPV, OpMNPV and AcMNPV.

	Nucleotide sequence	Amino acid sequence	
Comparison	Identity (%)	Identity (%)	Similarity (%)
SeMNPV with OpMNPV	36	39	55
SeMNPV with AcMNPV	38	26	44
AcMNPV with OpMNPV	53	42	62

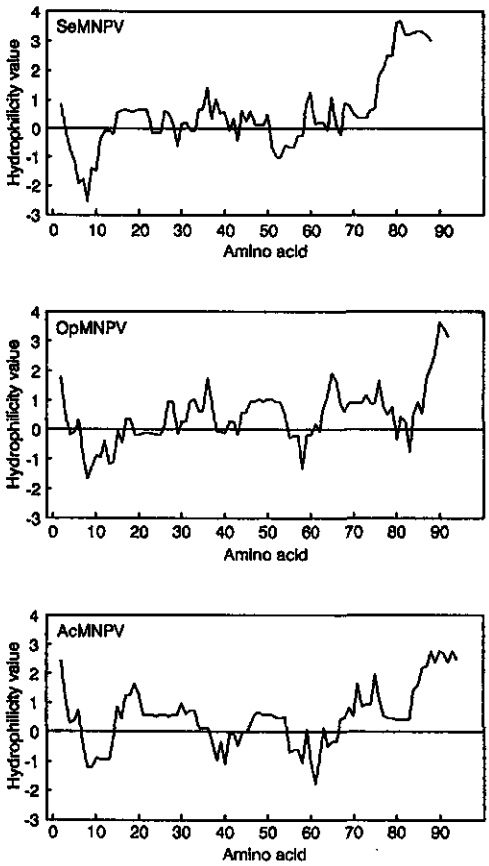

Figure 3.5. Hydrophilicity plots of the p10 proteins of SeMNPV, OpMNPV and AcMNPV. The methionine residue at the amino terminus of the p10 protein has been omitted. The plots were constructed using the algorithms of Kyte and Doolitle (1982), calculated with the PEPTIDE-STRUCTURE program of the UWGCG sequence analysis software package and plotted with LOTUS and FREELANCE programs (Lotus Development Corporation). The computer program plots the sums of hydrophilicity values for seven contiguous amino acids over the position of the middle amino acid in each sector.

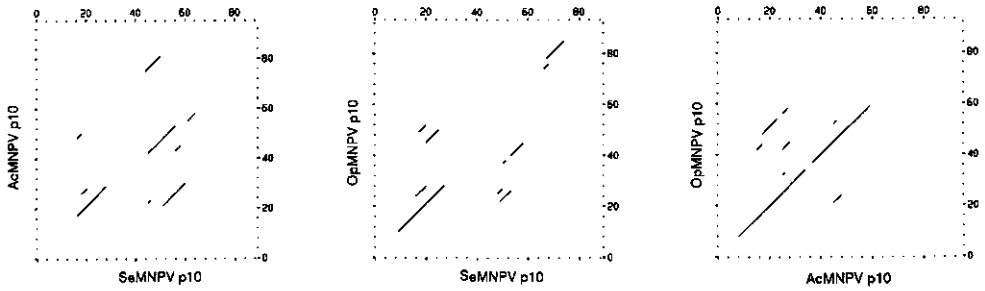

Figure 3.6. Dotplot comparisons of SeMNPV, OpMNPV and AcMNPV p10 proteins. The comparisons were performed with a window size of 21 and a stringency of 14 using the COMPARE and DOTPLOT programs of the GCG sequence analysis software package. The plots were generated with the FIGURE program of GCG.

DISCUSSION

This study describes the identification and characterization of the putative p10 gene of SeMNPV. The gene was mapped by Southern blot hybridization of digested SeMNPV DNA, using a cDNA probe derived from RNA isolated from SeMNPV-infected insect cells late after infection. The gene was located on the *Xba*I fragment H in the SeMNPV genome (Fig. 3.2) and is flanked by ORFs with homology to baculovirus p26 and p74 genes. This organization is collinear with the location of the p10 genes of AcMNPV (Liu *et al.*, 1986; Kuzio *et al.*, 1989) and OpMNPV (Bicknell *et al.*, 1986; Leisy *et al.*, 1986a). The limited degree of sequence homology between the p10 genes accounts for the failure to detect the SeMNPV p10 gene with an AcMNPV p10 gene probe. The abundance of p10 mRNA late after infection can be used to locate p10 genes of other baculoviruses with cDNA as probe. Alternatively, due to the conserved nature of p74 sequences and their apparent collinearity with p10 genes, p74 sequences can be used as probes to detect adjacent p10 genes.

The putative start site of the SeMNPV p10 transcripts probably maps within the sequence TAAG (Fig. 3.3). Transcription of most baculovirus late genes appear to involve the conserved TAAG motif (Blissard and Rohrmann, 1990). Recently, Zanotto *et al.* (1992) identified two putative structural elements conserved in the 5' non-coding region of all baculovirus polyhedrin genes sequenced to date. These elements share sequence homology and positional similarity within the 5' regulatory consensus regions of 5S ribosomal and

tRNA genes, which are involved in binding of class III transcription factors (Dillon, 1987; Lassar *et al.*, 1983). One of these elements, ATTGTA, was found in the 5' non-coding region of the SeMNPV p10 gene. A similar sequence, TTTGTA, exists in the promoter region of the AcMNPV p10 gene (Kuzio *et al.*, 1984). However, neither of these motifs was present in the promoter region of the OpMNPV p10 gene (Leisy *et al.*, 1986a). The relevance of these motifs for p10 gene expression therefore remains unclear.

Partial ORFs were detected upstream and downstream of the SeMNPV p10 gene and extended beyond the sequenced regions. The putative upstream ORF, which terminated 5 nt upstream of the ATAAG transcription initiation motif of the p10 gene, was orientated in the same direction as the p10 gene (Fig. 3.3). This partial ORF shared 55% and 53% amino acid similarity with the OpMNPV and AcMNPV p26 ORFs, respectively. Both AcMNPV and OpMNPV p26 coding sequences were located directly upstream of the p10 genes and in the same direction as the p10 ORFs. A perfect poly(A) signal (AATAAA) could not be detected between this putative p26 ORF and the p10 coding sequences, as was also the case for AcMNPV and OpMNPV. The SeMNPV p26 transcripts may coterminate with the p10 transcripts as is the case for AcMNPV and OpMNPV (Rankin *et al.*, 1986; Bicknell *et al.*, 1987).

The putative downstream ORF, which terminates at a TAA stop codon 26 nt downstream of the putative poly(A) signal sequence (AATAAA) of the p10 transcripts, is oriented in the opposite direction (Fig. 3.3). This partial ORF shares 77% and 71% amino acid similarity with the AcMNPV and OpMNPV p74 proteins, respectively (Kuzio *et al.*, 1989; Leisy *et al.*, 1986a). There is no putative poly(A) signal (AATAAA) detected between the p74 ORF and the p10 ORF in SeMNPV, as was also the case in AcMNPV (Kuzio *et al.*, 1989). However, a putative poly(A) signal was detected in this opposite direction just downstream of the p10 transcriptional start sequence (ATAAG) within the 5' non-coding region of the SeMNPV p10 gene (Fig. 3.3), a feature also shared by AcMNPV.

Recently the nucleotide sequence of the *Choristoneura fumiferana* MNPV (CfMNPV) p10 gene became available through the EMBL data library (accession no. M98513). Computer aided research revealed that the putative CfMNPV p10 protein contained 81 amino acids, was devoid of cysteine, histidine and tryptophan residues and had, like other p10 proteins, a highly basic carboxy terminus. The SeMNPV p10 protein shared an amino acid similarity of 37% with the CfMNPV p10 protein. The two conserved homology boxes (Fig. 3.4) were also present in the CfMNPV p10 coding sequence.

From the available data on the p10 genes of AcMNPV, OpMNPV and SeMNPV it can be postulated that the size of the ORF, and the structure and function of the protein it encodes are preserved among nuclear polyhedrosis viruses, whereas the nucleotide and amino acid sequence of the gene product are much more divergent than those of other baculovirus

genes (Rohrmann, 1992). This may suggest that the p10 gene has evolved from an ancestor gene more rapidly than other baculovirus genes.

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REFERENCES

Bicknell, J.N., Leisy, D.J., Rohrmann, G.F. and Beaudreau, G.S. (1987). Comparison of the p26 gene region of two baculoviruses. *Virology* 161: 589-592.

Birnstiel, M.L., Busslinger, M. and Strub, K. (1985). Transcription termination and 3' processing: the end is in site!. *Cell* 41: 349-359.

Blissard, G.W. and Rohrmann, G.F. (1990) Baculovirus diversity and molecular biology. *Annual Review of Entomology* 35: 127-155.

Caballero, P., Zuidema, D., Santiago-Alvarez, C. and Vlak, J.M. (1992). Biochemical and biological characterization of four isolates of *Spodoptera exigua* nuclear polyhedrosis virus. *Biocontrol Science and Technology* 2: 145-157.

Devereux, J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programmes for the VAX. *Nucleic Acids Research* 12: 387-395.

Dillon, L.S. (1987). The 5S ribosomal and other small RNAs. In *The gene: Structure, Function and Evolution.* pp 91-143. New York, Plenum Press.

Francki, R.I.B., Fauquet, C.M., Knudson, D. and Brown, F. (eds). (1991). Classification and nomenclature of viruses. Fifth Report of the International Commitee on Taxonomy of Viruses, *Archives of Virology Supplementum* 2: pp. 450.

Gelernter, W.D. and Federici, B.A. (1986a). Isolation, identification, and determination of virulence of a nuclear polyhedrosis virus from the beet army worm, *Spodoptera exigua* (*Lepidoptera*: *Noctuidae*). *Environmental Entomology* 15: 240-245.

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

Harrap, K.A. (1970). Cell infection by a nuclear polyhedrosis virus. *Virology* 42: 311-318.

Hink, F. (1970). Established insect cell line from the cabbage looper, *Trichoplusia ni*. *Nature (London)* 226: 466-467.

Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* 157: 105-132.

Kuzio, J., Jacques, R. and Faulkner, P. (1989). Identification of p74, a gene essential for virulence of baculovirus occlusion bodies. *Virology* 173: 759-763.

Kuzio, J., Rohel, D.Z., Curry, C.J., Krebs, A., Carstens, E.B. and Faulkner, P. (1984). Nucleotide sequence of the p10 polypeptide gene of *Autographa californica* nuclear polyhedrosis virus. *Virology* 139: 414-418.

Kuzio, J., Jaques, R. and Faulkner, P. (1989). Identification of p74, a gene essential for virulence of baculovirus occlusion bodies. *Virology* 173: 759-763.

Lassar, A.B., Martin, P.L. and Roeder, R.G. (1983). Transcription of class III genes: formation of preinitiation complexes. *Science* 222: 740-748.

Leisy, D.J., Rohrmann, G.F., Nesson, M. and Beaudreau, G.S. (1986a). Nucleotide sequence and transcriptonal mapping of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus p10 gene. *Virology* 153: 157-167.

Leisy, D.J., Rohrmann, G.F. and Beaudreau, G.S. (1986b). The nucleotide sequence of the polyhedrin gene region from the multicapsid baculovirus of *Orgyia pseudotsugata*. *Virology* 153: 280-288.

Liu, A., Qin, J., Rankin, C., Hardin, S.E. and Weaver, R.F. (1986). Nucleotide sequence of a portion of *Autographa californica* nuclear polyhedrosis virus genome containing the *Eco*RI site-rich region (hr5) and an open reading frame just 5' of the p10 gene. *Journal of General Virology* 67: 265-270.

Rankin, C., Ladin, B.F. and Weaver, R.F. (1986). Physical mapping of temporally regulated overlapping transcripts in the region of the 10K protein gene in *Autographa californica* nuclear polyhedrosis virus. *Journal of Virology* 57: 18-27.

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

Russell, R.L.Q., Pearson, M.N. and Rohrmann, G.F. (1991). Immunoelectron microscopic examination of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus-infected *Lymantria dispar* cells: time course and localization of major-polyhedron associated proteins. *Journal of General Virology* 72: 275-283.

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

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). Nucleotide sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* 74: 5463-5467.

Smith, G.E., Vlak, J.M. and Summers, M.D. (1982). *In vitro* translation of *Autographa californica* nuclear polyhedrosis virus early and late mRNAs. *Journal of Virology* 44: 199-208.

Smith, G.E., Vlak, J.M. and Summers, M.D. (1983). Physical analysis of *Autographa californica* nuclear polyhedrosis virus transcripts for polyhedrin and 10,000-molecular-weight protein. *Journal of Virology* 45: 215-225.

Smits, P.H., Van de Vree, M. and Vlak, J.M. (1988). Nuclear polyhedrosis virus for control of *Spodoptera exigua* on glasshouse crops. *Entomologia Experimentalis et Applicata* 43: 73-80.

Summers, M.D. and Arnott, H.J. (1969). Ultrastructural studies on inclusion formation and virus occlusion in nuclear polyhedrosis and granulosis virus-infected *Trichoplusia ni* (Hübner). *Journal of Ultrastructural Research* 28: 462-480.

Tautz, D. and Rentz, M. (1983). An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. *Analytical Biochemistry* 132: 14-19.

Van der Wilk, F., Van Lent, J.W.M. and Vlak, J.M. (1987). Immunogold detection of polyhedrin, p10 and virion antigens in *Autographa californica* nuclear polyhedrosis virus-infected *Spodotera frugiperda* cells. *Journal of General Virology* 68: 2615-2623.

Van Oers, M.M., Flipsen, J.T.M., Reusken, C.B.E.M., Sliwinsky, E.L., Goldbach, R.W. and Vlak, J.M. (1993). Functional domains of the p10 protein of *Autographa californica* nuclear polyhedrosis virus. *Journal of General Virology* 74: 563-574.

Van Strien, E.A., Zuidema, D., Goldbach, R.W. and 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.

Vlak, J.M. and Rohrmann, G.F. (1985). The nature of polyhedrin.In: *Viral Insecticides for Biological Control*, (K. Maramorasch and K.E. Sherman, eds), pp. 489-544, Academic Press, Orlando.

Williams, G.V., Rohel, D.Z., Kuzio, J. and Faulkner, P. (1989). A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *Journal of General Virology* 70: 187-202.

Zanotto, P.M.A., Sampaio, M.J.A., Johnson, D.W., Rocha, T.L. and Maruniak, J.E. (1992). The *Anticarsia gemmatalis* nuclear polyhedrosis virus polyhedrin gene region: sequence analysis, gene product and structural comparisons. *Journal of General Virology* 73: 1049-1056.

FUNCTIONAL DOMAINS OF THE *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS P10 PROTEIN

SUMMARY

Distinct functional domains in the *Autographa californica* nuclear polyhedrosis virus p10 protein were identified by analysis of p10 mutants. When up to fifteen amino acids from the carboxy terminus were deleted, truncated p10 proteins were observed in both the nucleus and the cytoplasm of infected cells, but fibrillar structures were not formed. This suggested that the positively charged carboxy terminus is not required for nuclear or cytoplasmic localization of p10, but is involved in protein-protein interactions leading to assembly of p10 into fibrillar structures. Absence of the p10 protein prevented the release of polyhedra from infected cells, due to impaired nuclear disintegration. This function of p10 appears to be located between amino acid residues 52 and 79. The amino-terminal half of p10 has already been implicated in the self-aggregation of this protein (Vlak *et al.*, 1988). Thus fibrillar structure formation, nuclear disintegration and intermolecular p10 protein interactions seem to be three separate functions of p10, located in distinct domains of the protein. The mutants expressing truncated p10 proteins were impaired in electron-dense spacer formation, but polyhedron envelopes were still formed. This result suggested that the formation of electron-dense spacers is not a prerequisite for the formation of polyhedron envelopes.

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INTRODUCTION

Infection of insect cells by nuclear polyhedrosis viruses (NPVs) (*Baculoviridae*; Francki *et al.*, 1991) is characterized by the synthesis of massive amounts of polyhedra (Rohrmann, 1986) and the formation of many rope-like structures, usually referred to as fibrillar structures (Van der Wilk *et al.*, 1987; Quant-Russell *et al.*, 1987). *Autographa californica* (*Ac*) MNPV and *Orgyia pseudotsugata* (*Op*) MNPV code for a protein of approximately 10 kDa, which is abundantly expressed in insect cells late after infection (Smith *et al.*, 1982; Rohel *et al.*, 1983; Quant-Russell *et al.*, 1987). In infected cells this protein, denoted as p10, is associated with the fibrillar structures. There is also evidence that p10 is associated with microtubules during infection (Volkman and Zaal, 1990). Fibrillar structures are present in the nucleus as well as in the cytoplasm of infected cells. In the nucleus these structures are often found in association with electron-dense spacers (MacKinnon *et al.*, 1973; Van Lent *et al.*, 1990; Quant-Russell *et al.*, 1991), suggesting a role of p10 in polyhedron morphogenesis.

Deletion mutagenesis of the p10 gene of AcMNPV showed that it is not essential for virus replication in cell cultures and in larvae. P10 deletion mutants were as infectious as wild type AcMNPV (Vlak *et al.*, 1990). These mutants, however, failed to liberate polyhedra from infected cells, suggesting a role of p10 in cell lysis (Williams *et al.*, 1989). An AcMNPV mutant expressing a fusion protein, consisting of the amino terminal 52 amino acids of p10 and *Escherichia coli* ß-galactosidase, induced granular structures in the nucleus, suggesting a bundling or cross-linking function as well as a nuclear import signal within the amino terminal half of the p10 protein (Vlak *et al.*, 1988).

The evidence suggests that the p10 protein, despite its limited size, has multiple functions in the infection process. To identify functional domains in the p10 protein and to study the possible role of these domains in the cytopathology of baculovirus infection, a series of carboxy-terminal p10 deletion mutants of AcMNPV were constructed and analysed. It appeared that the carboxy terminus is involved in the formation of fibrillar structures, but not in nuclear disintegration.

METHODS

Cell culture and virus

The *Spodoptera frugiperda* cell-line IPLB-SF-21 (Vaughn *et al.*, 1977) was used and maintained in plastic culture flasks in TNM-FH medium (Hink, 1970) supplemented with 10% foetal bovine serum (FBS). The multiple capsid form of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) strain E2 (Smith and Summers, 1978) was used as

wild-type (wt) virus. AcMNPV recombinant virus AcAS3 was used as a p10-negative control. This recombinant contained a deletion from +1 to +282 relative to the ATG, encompassing the complete p10 coding sequence (Vlak *et al.*, 1990).

Antisera

Antiserum prepared in rabbits against AcMNPV p10 protein, which had been purified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), was a gift from Dr. P. Faulkner (Queen's University, Kingston, Ontario, Canada). Antiserum prepared in rabbits against a fusion protein, containing the entire sequence of the polyhedron envelope (PE) protein (32 kDa) of *Orgyia pseudotsugata* (*Op*) MNPV fused to the trpE gene (Gombart *et al.*, 1989), was a gift from Dr. G.F. Rohrmann (Department of Agricultural Chemistry, Oregon State University, Corvallis, Oreg., USA).

Computer analysis

Hydrophilicity values, according to the methods of Kyte and Doolitle (1982), averaged over a window of seven amino acids and surface probability predictions (Emini *et al.*, 1985) were calculated for the AcMNPV p10 protein using the sequence data from Kuzio *et al.* (1984) with programs developed by the University of Wisconsin Genetics Computer Group (UWGCG). Profiles were plotted using LOTUS and FREELANCE programs (Lotus Developement Corporation).

Construction of AcMNPV p10 transfer vectors

AcMNPV transfer vectors were constructed with various deletions in the p10 coding sequence using standard molecular cloning techniques (Sambrook *et al.*, 1989). All transfer vectors were derived from pAcAS3 (Vlak *et al.*, 1990). The AcMNPV *Eco*RI-P clone pAcR159 (Smith *et al.*, 1983) was used as source for the p10 gene (Fig. 4.1 A).

Two transfer vectors, pAcMO13 and pAcMO14 (Fig. 4.1 B) were constructed by exploiting the internal *Hin*dIII and *Bgl*II restriction site, respectively. A 595 base pair (bp) *Eco*RI/*Hin*dIII fragment, containing the p10 gene up to the internal *Hin*dIII site preceded by upstream sequences, was isolated from the original *Eco*RI-P fragment in pAcR159 and cloned into pUC19 (pAcMO10). A synthetic self-complementary linker molecule with the sequence 5' AGCTTAAGGATCCTTA 3' was inserted into the *Hin*dIII site of pAcMO10 (pAcMO11), leading to the introduction of a translational stop codon and a *Bam*HI site immediately downstream of the GCT triplet, now coding for a carboxy-terminal alanine residue at position 79 in the amino acid sequence. In a similar way, a plasmid called pAcMO12 was constructed, by inserting a linker with the sequence 5' GATCTAAGGATCCTTA 3' into the *Bgl*II site of pAcMO10, resulting in a translational stop codon and a *Bam*HI site downstream

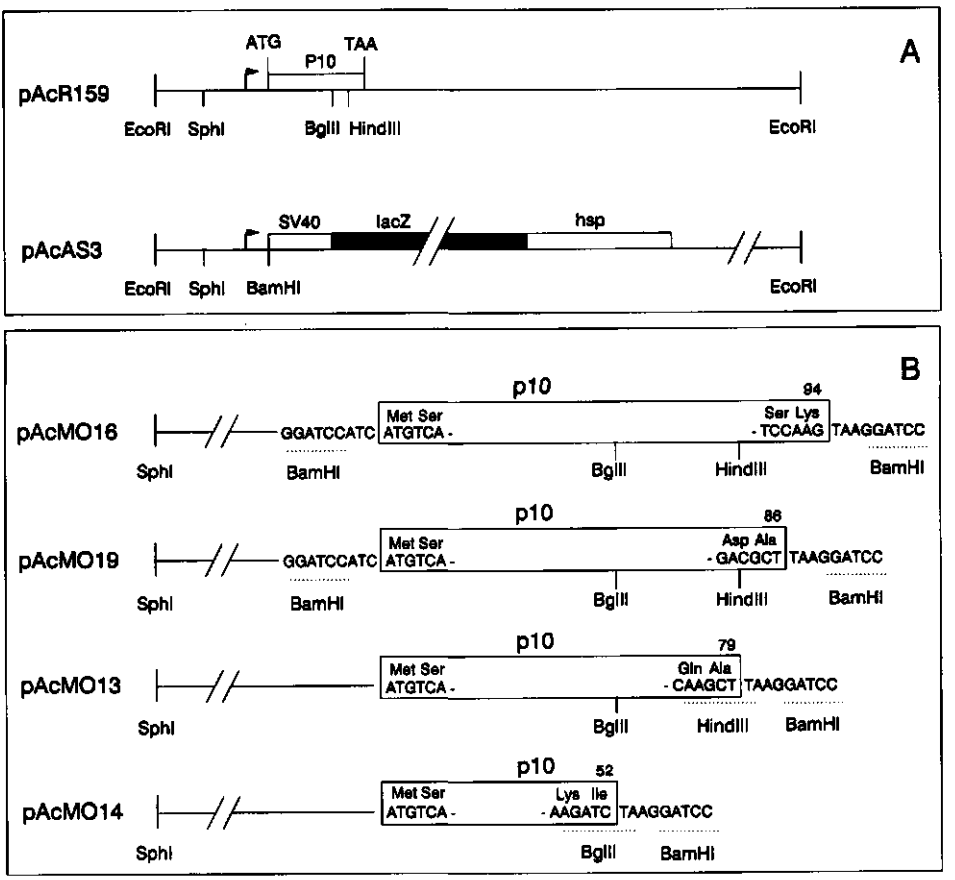

Figure 4.1. A) Schematic representation of the wt AcMNPV *Eco*RI-P fragment as cloned into pBR325 (pAcR159, Smith *et al.*, 1983) and of the transfer vector pAcAS3 (Vlak *et al.*, 1990) used as parent plasmids in the cloning experiments. Arrows mark the p10 mRNA start site. Only restriction sites used for cloning are indicated. B) Enlarged representation of the *Sph*I-*Bam*HI segment in the various transfer vectors, starting at the *Sph*I site (corresponding to nucleotide residue -230 in the authentic p10 gene) and ending at the *Bam*HI site downstream of the p10 coding sequence. Transfer vector pAcMO16, contains a full length p10 coding sequence, and the other transfer vectors are truncated at the indicated triplets. Apart from the *Sph*I-*Bam*HI segment, the transfer vectors are entirely comparable to pAcAS3.

of the ATC triplet coding for a carboxy-terminal isoleucine residue at position 52. From pAcMO11 and pAcMO12 *Sph*I-*Bam*HI fragments of 479 and 398 bps, respectively, were isolated and exchanged for the 230 bp *Sph*I-*Bam*HI fragment in pAcAS3, resulting in pAcMO13 and pAcMO14.

To construct the transfer vectors pAcMO16 and pAcMO19 (Fig. 4.1 B) the desired part of the p10 sequence was first synthesized as a *Bam*HI fragment using PCR-technology and then inserted into pAcAS3. For pAcMO16 the p10 sequence was synthesized from position -3 to +285, relative to the translational start codon, including the entire p10 coding sequence with the translational stop codon and the three base pairs preceding the p10 start codon in the authentic gene. To facilitate cloning of this p10 sequence, *Bam*HI sticky ends were synthesized at both ends of the molecule, by adding non-homologous sequences to the 5' ends of the PCR-primers. The sequence of the upstream primer was 5' GGGGATCCATCATG-TCAAAGCCTA 3', homologous with the coding strand from position -3 to +13. The downstream primer was complementary to the coding strand from position +285 to +270 and had the sequence 5' GGGGATCCTTACTTGGAACTGCGT 3'. The reaction product was first cloned as a *Bam*HI fragment into pUC19 (pAcMO15) and then recloned into pAcAS3, resulting in pAcMO16.

To construct pAcMO19 the same upstream primer was used as for pAcM016. The oligonucleotide 5' GGGGATCCTTAAGCGTCTGAATCGAGT 3' was used as downstream primer and was complementary to the coding strand from position +258 to +243. It contained additional non-homologous sequences to introduce an ochre stop codon followed by a *Bam*HI site, downstream of the GCT triplet coding for a carboxy-terminal alanine at position 86. The PCR product was isolated and cloned as a *Bam*HI fragment into pUC19 (pAcMO18). From pAcMO18 a 270 bp *Bam*HI fragment was isolated and inserted into the *Bam*HI site of pAcAS3, resulting in pAcMO19.

For PCR 0.1 pg of template DNA (pAc159R linearized with *Eco*RV) was used together with 50 pmol of each primer, 200 μM of each of the four deoxynucleotides, 10 μl PCR reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% (w/v) gelatin) and 2.5 units of *Taq* polymerase (Perkin Elmer Cetus) in a total volume of 100 μl. The reaction mixture was covered with 50 μl paraffin to prevent evaporation. Thirty cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C were carried out using a step-cycle device (Perkin-Elmer-Cetus). After chloroform extraction to remove paraffin, the DNA was precipitated and digested with *Bam*HI. The products were purified from a 1% agarose gel to remove primers and template DNA. DNA sequencing was performed using standard techniques on all PCR-derived clones. The insertions of p10-specific sequences into the transfer vector pAcAS3 was verified using the AcMNPV p10-specific primer 5' GAATTAT-TATCAAATCATTTG 3' homologous with the coding strand from position -68 to -48.

Transfection of viral DNA and isolation of recombinant viruses

Recombinant viruses were obtained by cotransfecting wt AcMNPV DNA with plasmid DNA, using the lipofectin method as described by Groebe *et al.* (1990). Recombinant viruses were selected as giving blue colouring plaques upon the addition of 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal, Boeringer Mannheim). Recombinant viruses were plaque purified (Brown and Faulkner, 1977) to obtain genetic homogenicity and grown into high titre stocks.

DNA and protein analysis of recombinant viruses

DNA was extracted from infected cells at 48 h post infection (p.i.) and the *Bam*HI restriction pattern was analysed in 0.7% agarose gels. For recombinants obtained by cotransfections with pAcMO16 and pAcMO19, Southern blot analysis (Sambrook *et al.*, 1989) was performed after *Bam*HI digestion using GeneScreenPlus™ membranes (Dupont) and a random primed ^{32}P-labelled p10-specific probe derived from a 294 bp *Bam*HI fragment isolated from pAcMO15.

SF-21 cells infected with wt AcMNPV or one of the recombinant viruses AcAS3, AcMO13, AcMO14, AcM016 and AcMO19 were harvested at 48 h p.i.and the protein pattern was analysed by SDS-PAGE according to Laemmli (1970) in a 13.5% gel using a Bio-Rad Mini-Protean^{II} apparatus. The gels were either stained with Coomassie brilliant blue or subjected to immunoblot analysis. On gels for immunoblotting rainbow markers (Amersham) were used to indicate the protein sizes.

Blots were treated for two hours at 37°C in 2% (w/v) fat-depleted milk powder in TBS (50 mM Tris-HCl, pH 7.4, 200 mM NaCl). Blots were then incubated for 1 h at room temperature with p10 antiserum (1:1000 diluted in 0.2% milk powder in TBS) or PE-antiserum (1:500 diluted). Blots were further incubated for 1 h with 1:1000 diluted goat-anti-rabbit immunoglobulins conjugated to alkaline phosphatase (Tago). Blots were stained in alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$) with 82.5 µg/ml nitroblue tetrazolium salt and 32.5 µg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (both from BRL Life Technologies).

RNA isolation and Northern blot analysis

Total RNA was isolated from infected SF-21 cells at 40 h p.i. and denatured as described by Van Strien *et al.* (1992). Eight micrograms of total RNA or 2.5 µg of RNA-ladder (BRL Life Technologies) were resolved in 1.4% agarose gels in 10 mM sodium phosphate buffer, pH 6.5-7.0. The RNA was transferred to the nylon membrane Hybond™-N (Amersham) in 25 mM sodium phosphate buffer and hybridized using standard techniques (Sambrook *et al.*, 1989) to a random primed ^{32}P-labelled p10-specific probe. This probe was derived from an

157 bp *Eco*RI/*Bgl*II fragment, isolated from plasmid pAcMO17. This plasmid was constructed by cloning the p10 coding sequence as an *Eco*RI/*Bam*HI fragment made by PCR into pUC19. For this PCR the upstream primer 5' GGGAATTCATGTCAAAGCCTAA 3' was used, giving an *Eco*RI site at the 5' end of the p10 sequence.

Electron microscopy and immunogold labelling

SF-21 cells were infected with wt AcMNPV, the control virus AcMO16 or one of the p10 deletion mutants at a multiplicity of infection (m.o.i.) of 10 $TCID_{50}$ units/cell. Cells were harvested 46 h p.i., fixed and embedded in LR Gold resin (London Resins Company) as described by Van Lent *et al.* (1990) with some modifications. Cells were fixed in 2% (w/v) paraformaldehyde, 3% (w/v) glutaraldehyde in PC buffer (0.1 M $Na_2HPO_4.2H_2O$, 9.7 mM citric acid in H_2O; pH 7.2) containing 1.5 mM $CaCl_2$ for 3 h at 4°C, washed twice in PC buffer and resuspended in 5% (w/v) gelatine in PC buffer.

Ultrathin sections were cut with a diamond knife using the Reichert-Jung Ultracut^E microtome and mounted on nickel grids coated with a formvar supporting film. Immunogold labelling was performed as described by Van Lent *et al.* (1990) with 1:1000 diluted p10 antiserum and subsequently with protein A-gold with a diameter of 7 nm. To study the distribution of p10 at lower magnifications silver enhancement was performed for 2 min at room temperature using the Aurion R-gent staining kit (Aurion, Wageningen, The Netherlands). Immunolabelled sections were stained as described by Flipsen *et al.* (1992) before examination in a Philips CM12 transmission electron microscope.

Phase-contrast microscopy and lactate dehydrogenase (LDH) assays to study cell lysis

One-million SF-21 cells were seeded in culture dishes and infected at a m.o.i. of 10 with wt AcMNPV and the various recombinants. After 90 min of incubation the inoculum was removed and cells were washed with TNM-FH medium supplemented with 10% FBS. Cells were covered with a thin agar overlay of 1.5% agarose in this medium. At 96 h p.i. cells were examined by phase contrast microscopy using a Leitz Labovert FS microscope.

For LDH assays 1.5 x 10^6 cells adjusted to the protein-free Sf900-II culture medium (Gibco-BRL) were inoculated with wt AcMNPV and AcAS3 at a m.o.i of 10. After 60 min of incubation, cells were washed once and covered with 2 ml Sf900-II medium and further incubated for 0, 48, 72 or 96 h. For each time point dual infections were performed. At each time point a sample of the culture medium was taken and filtered. The cells were resuspended in the remaining medium and sonicated at 35 Watt for 15 s using a sonic disrupter (type B12; Branson Sonic Power Company, CN, USA). The measurement of LDH activity in both fractions was based upon the conversion of pyruvate and NADH into L-lactate and NAD^+. The reaction was performed at 25°C in 50 mM phosphate buffer, pH 7.5,

1.8 mM sodium pyruvate, 0.13 mM NADH (both from Boehringer Mannheim), as recommended for *S. frugiperda* cells by Wu *et al.* (1990). The reaction was followed spectrophotometrically for 3 min by measuring the decreasing absorbance at 340 nm. The LDH activity was calculated from the decrease in absorbance (and thus in NADH-concentration) as recommended by Boehringer Mannheim. One unit LDH will convert 1 μmol of pyruvate to L-lactate in 1 min at 25°C and pH 7.5. The LDH activity in the medium was calculated as a fraction of the total LDH activity in cells and medium.

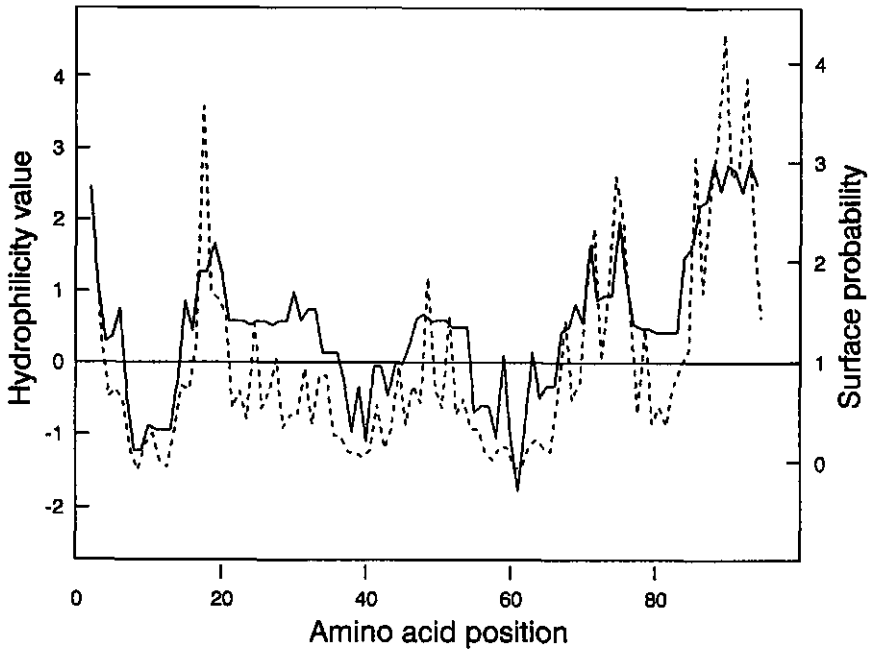

Figure 4.2. Hydrophilicity values (continious line) and surface probability predictions (dotted line) for the AcMNPV p10 protein, based on the sequence data of Kuzio *et al.* (1984) and the methods of Kyte and Doolitle (1982), and Emini *et al.* (1985), were calculated with the program PEPTIDESTRUCTURE from the UWGCG computer software package. They were plotted using LOTUS and FREELANCE programs (Lotus Development Corporation). The methionine at position 1 has been omitted. The hydrophilicity values were calculated over a window of 7 amino acids, positive values indicate hydrophilicity, negative values hydrophobicity. The surface probability values were calculated over a window of 6 amino acids. The averaged value for a random hexapeptide sequence is 1.0. Values above 1.0 indicate an increased probability for being found at the surface of a protein.

RESULTS

Prediction of functional domains in AcMNPV p10

The p10 proteins of AcMNPV, OpMNPV and SeMNPV share only limited amino acid sequence identity (Kuzio *et al.*, 1984; Leisy *et al.*, 1986; Zuidema *et al.*, 1993) with values varying from 26 to 42%. Despite these low amino acid homology, hydrophilicity plots (see also Chapter 3, Fig. 3.6) have revealed that the distribution of hydrophobic and hydrophilic amino acid residues is conserved in p10 proteins, although some variation exists. The most characteristic feature is the high hydrophilicity of the carboxy terminus, caused by the presence of several positively charged amino acids. Surface probability predictions indicated, that this hydrophilic carboxy terminus is most likely exposed at the surface of the protein (Fig. 4.2). Furthermore, the amino terminal half of both p10 proteins contained a hydrophobic region. The aggregation of amino-terminal p10-ß-galactosidase fusion proteins (Vlak *et al.*, 1988) in the nucleus suggests a bundling or cross-linking function for this region.

For further analysis of these possible domains p10 mutants were constructed (Fig. 4.1) with carboxy-terminal deletions of variable length, and characterized in terms of protein synthesis, intracellular location of p10 and cytopathological effect.

Construction of AcMNPV p10 mutants

To obtain AcMNPV mutants with deletions in the p10 coding sequence, several transfer vectors were constructed. Plasmid pAcAS3, a p10-promoter based transfer vector (Vlak *et al.*, 1990), was chosen as parental vector. This vector contains a gene cassette consisting of the *Drosophila melanogaster* hsp70 promoter, the *E. coli* ß-galactosidase coding sequence (lacZ) and the SV40 terminator sequence immediately downstream of the *Bam*HI site (Fig. 4.1 A). The presence of this cassette enabled convenient selection of recombinant viral plaques by virtue of the activity of ß-galactosidase upon addition of the chromogenic substrate X-gal.

Three AcMNPV mutants, varying in the extent of the carboxy-terminal p10 deletion (AcMO14, AcMO13 and AcMO19) and a control virus with a full length p10 sequence (AcMO16) were obtained by recombination of wt AcMNPV DNA with each of the transfer vectors (Fig. 4.1 B). The recombinant virus AcAS3, lacking the entire p10 coding sequence (Vlak *et al.*, 1990), served as a further control. After plaque purification the identity of recombinant viruses was confirmed by restriction enzyme analysis. In AcMO19 the triplets coding for the 8 mainly basic carboxy-terminal amino acids were deleted. In AcMO13 the deletion extended to the last 15 triplets and in AcMO14 the triplets coding for the carboxy-terminal half of p10 (42 amino acids) were removed. In each mutant an in-frame TAA stop

codon was introduced to prevent the synthesis of additional amino acids at the carboxy terminus of the truncated p10 proteins. Recombinant AcMO16, carrying the complete p10 coding sequence was used as a positive control as it contained the same restriction sites in the up- and downstream regions as the deletion mutants, in addition to the lacZ cassette. In both AcMO16 and AcMO19 *Bam*HI sites were present on both sites of the p10 coding sequence as was confirmed by restriction endonuclease and Southern analysis.

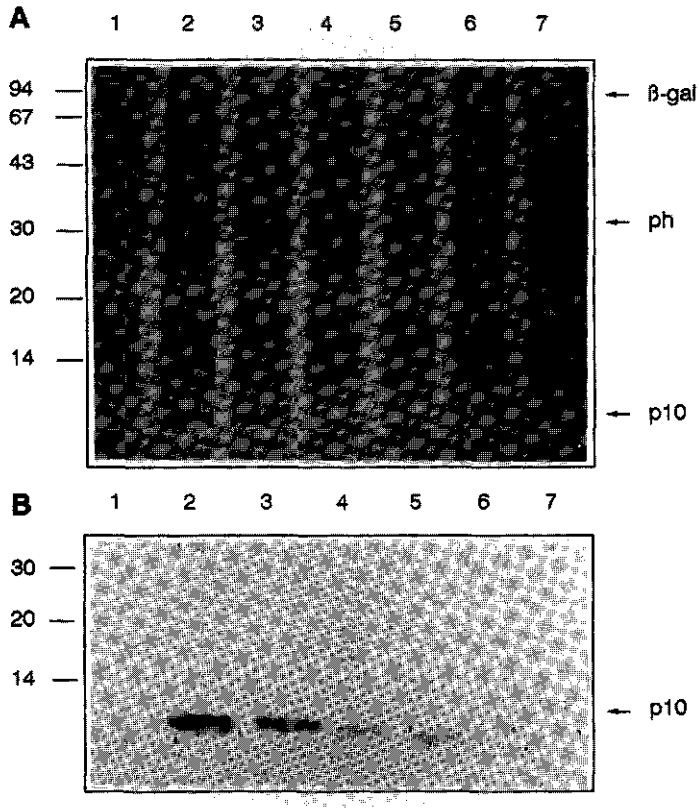

Figure 4.3. A) *S. frugiperda* cells (1) infected with wt AcMNPV (2) and the various recombinants AcMO16 (3), AcMO19 (4), AcMO13 (5), AcMO14 (6) and AcAS3 (7) were harvested 48 h post infection and analysed in a 13,5% SDS-polyacrylamide gel. B) Following gel electrophoresis the proteins were transferred to nitrocellulose and Western analysis was performed using p10 polyclonal anti-serum. Each lane contained the equivalent of 4.7 x 10^4 cells. Molecular weight markers are indicated (in 10^3 kDa). as are ß-galactosidase (ß-gal), polyhedrin (ph) and p10 protein (p10).

Expression of mutant p10 genes

To analyse the expression of truncated p10 proteins for the various recombinant viruses, infected cells were analysed by SDS-PAGE at 48 h p.i. (Fig. 4.3 A). Cells infected with the control recombinant AcMO16 (lane 3) showed a p10 protein of the same size as wt AcMNPV infected cells (lane 2) and in equivalent amounts. Thus, the expression of p10 was not perturbed by the introduction of a *Bam*HI site in the leader sequence, nor did the presence of the SV40 terminator, as a result from the insertion of the lacZ marker cassette, have any noticeable adverse effect.

Cells infected with the deletion mutants AcMO19 (Fig. 4.3 A, lane 4) or AcMO13 (lane 5) produced truncated p10 proteins of the size predicted from the introduced deletion. Their identity as p10-derived polypeptides was confirmed by Western analysis using p10-specific polyclonal rabbit antiserum (Fig. 4.3 B, lane 4 and 5). In cells infected with AcMO14 (lane 6) no p10-related protein could be detected, neither by SDS-PAGE nor by Western analysis. This was found for two independently isolated, plaque-purified recombinants. In accordance with previous results (Vlak *et al.*, 1990), AcAS3-infected cells (lane 7) did not produce the p10 protein.

Transcriptional analysis of p10 deletion mutants

To investigate the transcriptional activity of the truncated p10 genes in the various mutants, Northern blot analysis was performed on total RNA isolated at 40 h p.i. (Fig. 4.4). In wt AcMNPV infected cells (Fig. 4.4; lane 2) p10-specific transcripts of approximately 750

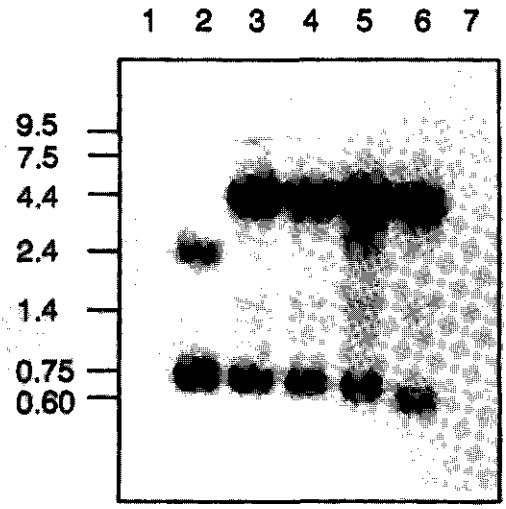

Figure 4.4. Total RNA was isolated at 40 h post infection from *S. frugiperda* cells (1) infected with wt AcMNPV (2) and the various recombinants AcMO16 (3), AcMO19 (4), AcMO13 (5), AcMO14 (6) and AcAS3 (7) and analysed by Northern blot hybridization with an AcMNPV p10 specific probe. Each lane contained 8 μg of RNA. RNA markers are indicated in kb.

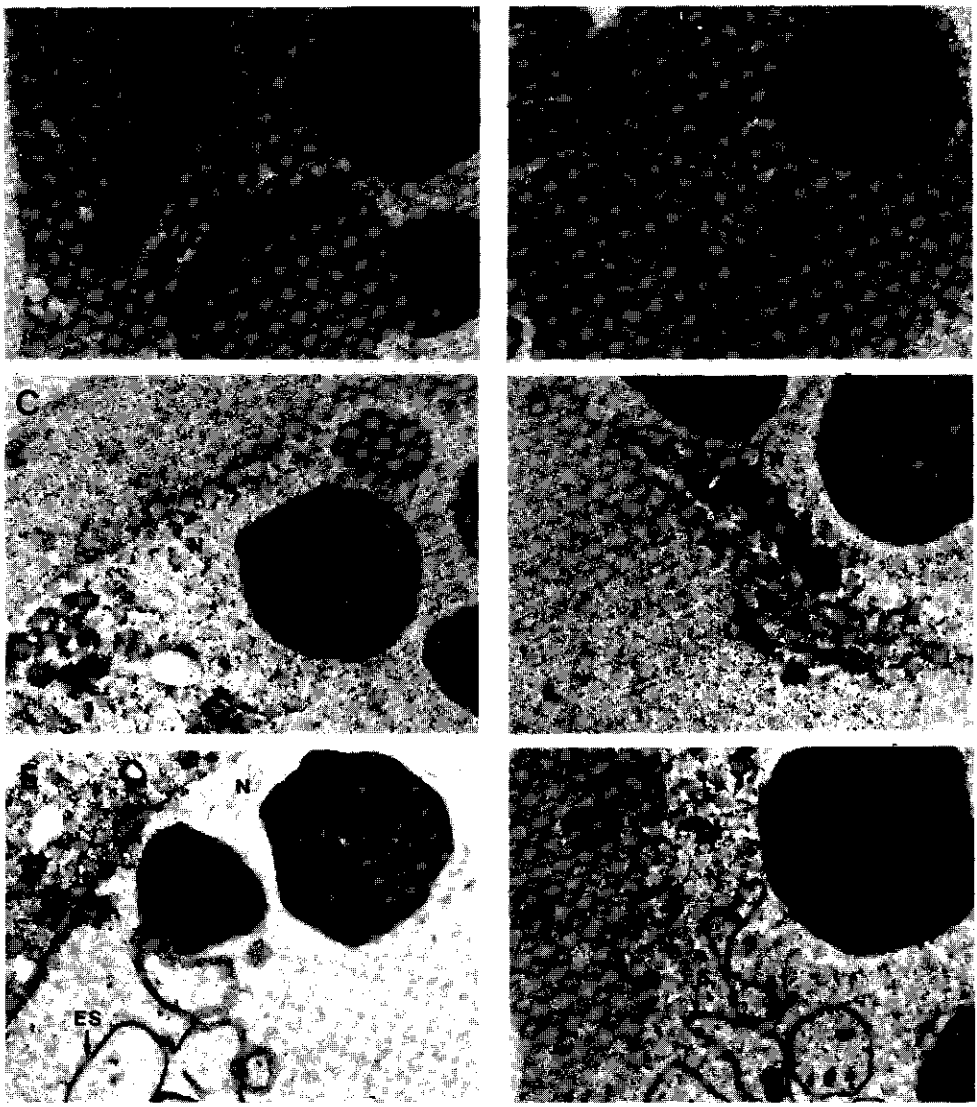

Figure 4.5. Electron micrographs of thin sections of *S. frugiperda* cells infected with wt AcMNPV (A), AcMO16 (B), AcMO19 (C), AcMO13 (D), AcMO14 (E) and AcAS3 (F) and harvested at 46 h p.i. The sections were treated with antiserum and subsequently complexed to Protein A-gold to detect p10 protein. Fibrillar structures (F) and electron-dense spacers (ES) are indicated, as well as the nucleus (N) and the cytoplasm (C). The bar marker represents 0.5 μm.

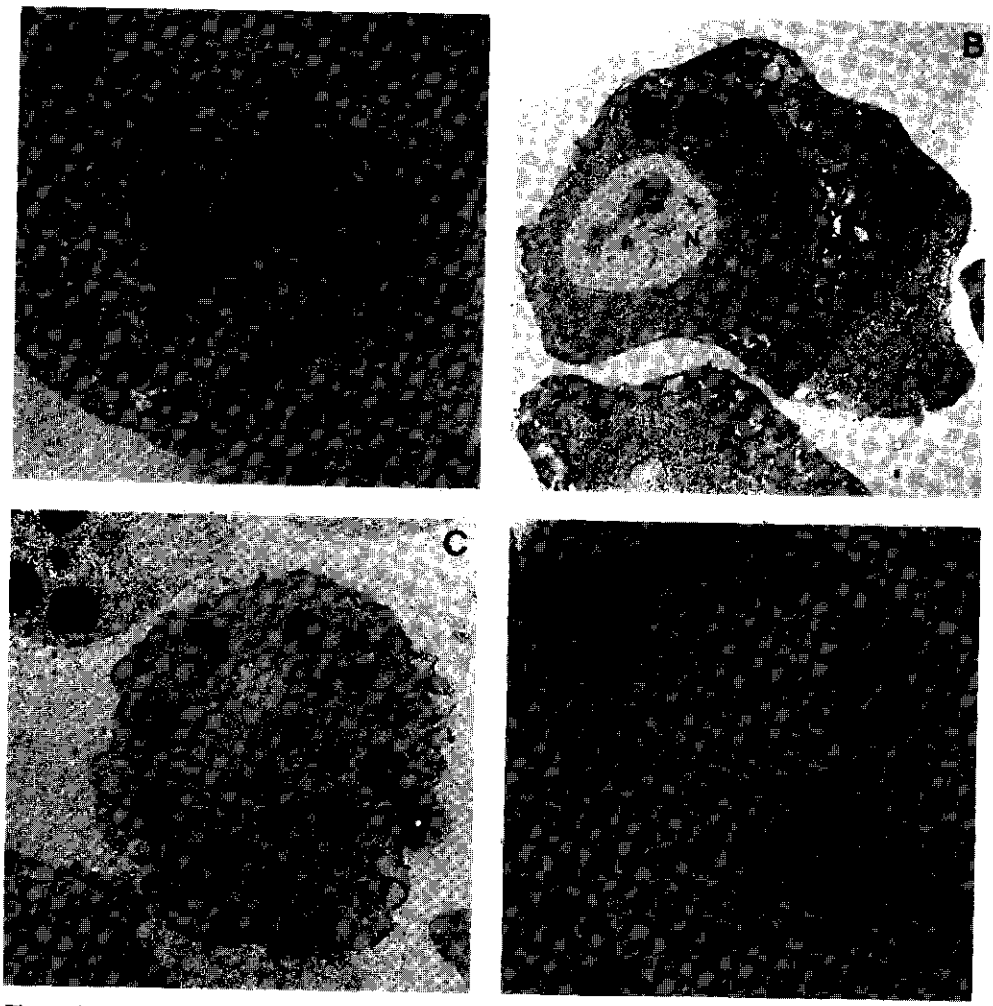

Figure 4.6. Electron micrographs of thin sections of *S. frugiperda* cells infected with *wt* AcMNPV (A), AcMO19 (B), AcMO13 (C) and AcAS3 (D) and harvested at 46 h p.i. The sections were subsequently treated with antiserum, complexed to Protein A-gold and subjected to silver enhancement to detect p10 protein. The nucleus (N) and cytoplasm (C) are indicated. The bar marker represents 2.5 μm.

and 2500 nucleotides (nt) were observed. This is in agreement with the experiments of Rankin *et al.* (1986), who reported two late transcripts of these sizes, originating from the p10 promoter but terminating at different positions. With the control recombinant AcMO16 (lane 3) a transcript of approximately 700 nt was observed, as expected for a transcript starting at the p10 promoter and terminating at the SV40 terminator. With the deletion mutants AcMO19 (lane 4), AcMO13 (lane 5) and AcMO14 (lane 6) p10-specific transcripts of decreasing size were observed equivalent to the length of the truncated p10 sequence.

In cells infected with recombinants AcMO16 (Fig. 4.4, lane 3), AcMO19 (lane 4), AcMO13 (lane 5) or AcMO14 (lane 6) a longer transcript of approximately 4400 nt also hybridized to the probe. This transcript most likely originated from the p10 promoter, but failed to terminate at the SV40 terminator, passed through the lacZ gene and the hsp70 fragment, and terminated at the authentic p10 transcriptional stop signal. Comparable read-through transcripts have also been observed with other recombinants in which the SV40 terminator was exploited (Roelvink *et al.*, 1992).

Cytopathology of p10 deletion mutants

To study the cytopathological effects of p10-deletion mutants, cells were collected 46 h p.i. and processed for electron microscopy. The p10 polypeptides were detected using a p10 antiserum and colloidal gold conjugated to Protein A. In cells infected with mutant AcMO16 (Fig. 4.5 B), producing a wt p10 protein, normal fibrillar structures were found and no differences in cytopathology as compared to the wt AcMNPV (Fig. 4.5 A) were observed. In contrast, the various deletion mutants were all deficient in the formation of fibrillar structures (Fig. 4.5 C to F). In AcMO14-infected cells (Fig. 4.5 E) the truncated p10 protein was not detected, consistent with our inability to detect it by Western analysis (Fig. 4.5, lane 6). In AcMO13- and AcMO19-infected cells, p10-specific labelling was observed in both the cytoplasm and the nucleus, as can clearly be seen upon silver enhancement of immunogold-labelled sections (Fig. 4.6 B and C). The p10 polypeptides were not homogeneously dispersed throughout these compartments. For both AcMO13 and AcMO19 the truncated p10 proteins in the nucleus were not associated with the virogenic stroma, chromatin or polyhedra, whereas in the cytoplasm the p10 protein occurred in defined areas. In these areas no large organelles such as mitochondria or vesicles were present. Ribosomes, however, were present in these areas.

Cells infected with AcMO19 and AcMO13, which do not express the carboxy-terminal hydrophilic domain, did not contain electron-dense spacers (Fig. 4.5 C and D), although the polyhedra were surrounded by envelopes. This was observed with two independently-isolated recombinants of both AcMO19 and AcMO13. Spacer-like structures were only sometimes observed in disrupted cells. On the other hand, mutants AcMO14 and AcAS3, did induce

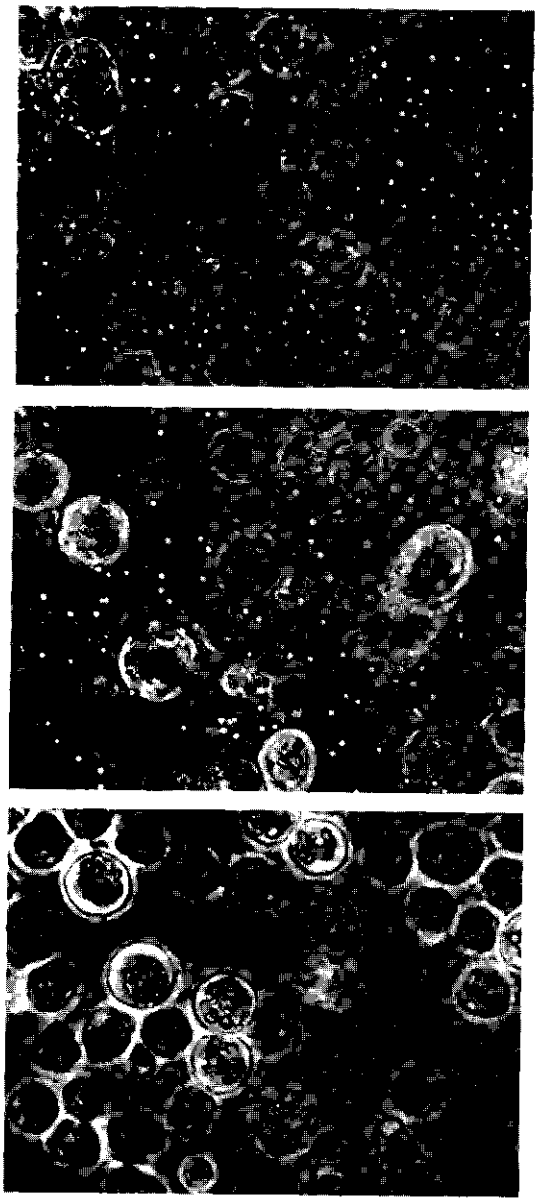

Figure 4.7. Phase contrast images of *S. frugiperda* cell cultures infected with wt AcMNPV (A), AcMO13 (B), and AcMO14 (C) at 96 h p.i. The bar marker represents 20 μm.

electron-dense spacers (Fig. 4.5 E and F). SDS-PAGE and immunoblot analysis revealed that the polyhedron envelope protein was synthesized in quantities comparable to wt AcMNPV for all recombinants (data not shown).

Release of polyhedra

Since a lack of p10 protein in AcMNPV infected cells was found to be associated with impaired release of polyhedra, it was postulated that the protein had a role in cell lysis (Williams *et al.* 1989). To investigate whether carboxy-terminal deletions in p10 have any effect on cell lysis, cells infected with either wt AcMNPV or the mutants viruses were studied by phase contrast microscopy. Infection with wt AcMNPV (Fig. 4.7 A), the control AcMO16 and the deletion mutants AcMO19 and AcMO13 (Fig. 4.7 B), led to high levels of polyhedra in the culture medium at 96 h p.i. During infection with AcMO14 (Fig. 4.7 C) and AcAS3, polyhedra were retained inside the nucleus of infected cells. Thus, mutants that produced a p10 protein lacking the carboxy-terminal 8 or 15 amino acids and, which cosequently, did not induce fibrillar structures, were still able to release polyhedra. This function was abolished in cells infected with AcMO14 and AcAS3, in which no p10 specific protein was observed.

To determine whether release of polyhedra in AcMO14- and AcAS3-infected cells was impaired by an inhibited cell lysis or by an inability of the nuclei to disintegrate, the presence of LDH in the culture medium, a marker for cytoplasmic disintegration, was followed over time for wt AcMNPV- and AcAS3-infected cells. At the start of the post infection period no measurable LDH activity was present in the medium. At 48 h p.i. the LDH activity was less than 5% of the total activity in cells plus medium. At 72 h p.i. the LDH activity in the medium was about 15%, and at 96 h p.i. 25% for both wild type and AcAS3 infected cells. In AcAS3-infected cells the cytoplasmic membrane appears to disintegrates, while the nucleus remains intact.

DISCUSSION

P10 is an abundant viral protein in baculovirus-infected cells and it is found associated with fibrillar structures in the nucleus and cytoplasm of these cells (Van der Wilk *et al.*, 1987; Quant-Russell *et al.*, 1987). Other functions of p10 such as in cellular or nuclear lysis (William *et al.*, 1989), in bundling or cross-linking microtubules (Volkman and Zaal, 1990) or polyhedron envelope morphogenesis (Vlak *et al.*, 1988; Quant-Russel *et al.*, 1991), are somewhat enigmatic. In this paper, by using deletion mutagenesis of AcMNPV p10, we provide evidence for the existence of at least two functional domains on the p10 protein. The results of the analyses are summarized in Table 4.1.

Table 4.1. Biological characteristics of wt AcMNPV and the various p10 deletion mutants.

Virus	P10 specific protein	P10 specific transcripts	Fibrillar structures	Polyhedron envelope	Electron-dense spacers	Polyhedron release
wt AcMNPV	+	+	+	+	+	+
AcMO16	+	+	+	+	+	+
AcMO19	+	+	-	+	-	+
AcMO13	+	+	-	+	-	+
AcMO14	-	+	-	+	+	-
AcAS3	-	-	-	+	+	-

P10 molecules lacking 8 carboxy-terminal amino acid residues (AcMO19) failed to assemble into fibrillar structures. The positively charged carboxy terminus might be involved in intermolecular protein-protein interactions between p10 molecules, or with host cell components such as microtubules. Cross-reactivity of a monoclonal antibody against OpMNPV p10 with microtubules in uninfected cells indeed indicated sequence homology between this p10 and a non-tubulin component of microtubules (Quant-Russell *et al.*, 1987). The cross-reacting epitope was localized in the carboxy-terminal half of the OpMNPV p10. Recent work has indicated that phosphorylation of the carboxy-terminus of p10 upon expression of a non-baculovirus serine-kinase induced binding of p10 to microtubules (Cheley *et al.*, 1992). Our data support the hypothesis that the carboxy terminus is involved in interaction with microtubules. Site-specific mutagenesis may indicate which of the carboxy-terminal amino acid residues is involved in this process.

Although fibrillar structures were absent from cells infected with AcMO19 and AcMO13, expressing p10 lacking 8 and 15 amino acids from the carboxy terminus, release of polyhedra was still observed. In AcAS3- and AcMO14-infections polyhedra remained occluded in the nuclei of infected cells, although the cytoplasm disintegrated. This indicates the existence of separate domains of p10 involved in the release of polyhedra and in the formation of fibrillar structures. From the LDH release measurements it was concluded that nuclear disintegration, leading to release of polyhedra, is a process separate from virus induced cell lysis. Polyhedra were not released from cells infected with a p10-ß-galactosidase fusion mutant (Williams *et al.*, 1989), but were from AcMO13-infected cells, suggesting a function of the sequence between 52 and 79 in the disintegration of nuclei. In this region in the AcMNPV p10 protein the sequence VPDLP is found between amino acid residues 64 and

68. In OpMNPV p10 homologous sequences are found between residues 77 and 81 (LPEIP), and 80 and 84 (IPDVP). This part of the protein will be further analysed by introducing point mutations.

Since amino-terminal fusions of p10 with ß-galactosidase were able to aggregate in the nucleus of mutant AcMNPV-infected cells (Vlak *et al.*, 1988) and the truncated p10 proteins described here (AcMO19 and AcMO13) show localization to confined areas in nucleus and cytoplasm (Fig. 4.5 and Fig. 4.6), we postulate that the amino-terminal half of p10, is involved in the supramolecular organization of p10 molecules.

The expression of truncated p10 molecules, lacking 8 or 15 amino acids, prevented electron-dense spacer formation, although polyhedron envelopes were still formed and the PE protein was synthesized. This protein is a structural component of both electron-dense spacers and polyhedron envelopes (Whitt and Manning, 1988; Zuidema *et al.*, 1989; Van Lent *et al.*, 1990; Quant-Russell and Rohrmann, 1990). The role of electron-dense spacers is only speculative, but it was postulated that they represent precursors of the polyhedron envelope (Vlak *et al.*, 1988). However, from the experiments described here (Table 4.1), it may be concluded that electron-dense spacers are not necessary for the formation of polyhedron envelopes, and that they, therefore, do not necessarily represent early stages in the development of polyhedron envelopes. The mutants, that contained a larger (AcMO14) or a complete (AcAS3) deletion of the p10 coding sequence, induced normal electron-dense spacers and envelopes surrounded the polyhedra. Furthermore, in an AcMNPV recombinant, in which the p10 coding sequence was replaced by the lacZ sequence, the capacity to form electron-dense spacers and polyhedron envelopes was also maintained (Van Lent *et al.*, 1990). It seems that p10 is not essential for the formation of polyhedron envelopes.

A possible explanation for the absence of electron-dense spacers in AcMNPV recombinants expressing truncated p10 proteins may be, that p10 has a certain affinity for a component of the electron-dense spacers, perhaps the PE protein. Fibrillar structures are often found in perpendicular association with electron-dense spacers or polyhedron envelopes, suggesting the existence of such binding sites. In wt AcMNPV-infected cells p10 is captured in fibrillar structures leaving only a small number of binding sites available for interaction with the PE protein. When the carboxy terminus of p10 is deleted and fibrillar structures are not formed any longer, many binding sites become available for the PE protein, resulting in a reduced concentration of free PE protein and thus preventing electron-dense spacer formation. Since polyhedron envelopes are still formed, polyhedrin molecules appear to have a higher affinity for PE protein than p10. When the complete p10 sequence was deleted (AcAS3) or when no p10 protein could be detected (AcMO14) electron-dense spacers were formed, since this putative binding site is absent.

Mutant AcMO14, did not express detectable amounts of p10 protein, although transcripts were present. The failure to detect this truncated p10 protein on Western blots and in thin sections might be caused by instability and hence by rapid degradation of this small polypeptide (52 amino acids), or by the absence of epitopes recognized by the antiserum. Mutant AcMO14 behaved phenotypically like AcAS3, in which the complete p10 gene open reading frame was deleted. An other AcMNPV p10 mutant studied by Williams *et al.* (1989), in which only the 29 amino-terminal amino acids were retained, did not show any detectable truncated p10 protein, either.

In cells infected with p10-ß-galactosidase fusion mutants polyhedron envelopes were absent (Vlak *et al.*, 1988; Williams *et al.*, 1989), although spacers were described in the latter paper. The reason for the discrepancy between the two papers remains unclear. It was postulated that the recombinant made by Vlak *et al.* (1988) had an additional mutation (Van Lent *et al.*, 1990), but we also noticed that the two fusion proteins were not exactly identical. The protein found by Williams *et al.* (1989) had an additional proline between the p10 and ß-galactosidase derived parts, possibly leading to altered characteristics of the fusion protein. The absence of polyhedron envelopes in both fusion mutants is most likely the result of a specific characteristic of the fusion protein, since mutants expressing high levels of ß-galactosidase are perfectly capable of forming envelopes.

Since the p10-ß-galactosidase fusion protein is capable of assembling into granular structures in the nucleus, it has been postulated that the amino-terminal part contained a nuclear import signal (Vlak *et al.*, 1988). However, this conclusion may be premature, since a p10-deletion mutant expressing a non-fused lacZ gene from the p10 promoter revealed ß-galactosidase in both nuclei and cytoplasm of infected cells (Van Oers *et al.*, 1990). In view of the size of the AcMNPV p10 protein and, since it is localized in both nucleus and cytoplasm, it is not clear whether its transport to the nucleus involves a nuclear import signal. The localization of p10 in both compartments may point to passive transport by diffusion. Alternatively, the incorporation of p10 into fibrillar structures, might prevent its complete import into the nucleus. The formation of fibrillar structures in the nucleus might, in turn, prevent diffusion of p10 into the cytoplasm. Small proteins have been reported to enter the nucleus by diffusion when microinjected into the cytoplasm (Bonner, 1975). However, histones and other small nucleophilic proteins, appear to be targeted to the nucleus by an energy requiring mechanism involving binding to a cytoplasmic receptor (Breeuwer and Goldfarb, 1990). The small structural proteins Vp2 and Vp3 (38 and 27 kDa) of SV40, for instance, harbour their own nuclear import signals (Clever and Kasamatsu, 1991). For the AcMNPV polyhedrin protein (33 kDa) a nuclear targeting signal has been determined on the positively charged, internal sequence KRKK (Jarvis *et al.*, 1991). The sequence of the carboxy terminus of AcMNPV p10 is RRGKRSSK (Kuzio *et al.*, 1984) and resembles

identified nuclear targeting signals (Pugsley, 1989). However, the truncated p10 molecules expressed by recombinant AcMO19, in which this sequence was deleted, were localized in both the cytoplasm and the nucleus, indicating that the basic carboxy-terminal sequence is not essential for nuclear import. The truncated p10 proteins of AcMO13, in which an additional stretch of seven carboxy-terminal amino acids (FELDSDA) were deleted, exhibited a similar localization.

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REFERENCES

Bonner, W.M. (1975). Protein migration into nuclei. I. Frog oocyte nuclei *in vivo* accumulate microinjected histones, allow entry to small proteins, and exclude large proteins. *Journal of Cell Biology* 64: 421-430.

Breeuwer, M. and Goldfarb, D.S. (1990). Facilitated transport of histone H1 and other small nucleophilic proteins. *Cell* 60: 999-1008.

Brown, M. and Faulkner, P. (1977). A plaque assay for nuclear polyhedrosis viruses using a solid overlay. *Journal of General Virology* 36: 361-364.

Cheley, S., Kosik, K.S., Paskevich, P., Bakalis, S. and Bayley, H. (1992). Phosphorylated baculovirus p10 is a heat-stable microtubule-associated protein associated with process formation in Sf9 cells. *Journal of Cell Science* 102: 739-752.

Clever, J. and Kasamatsu, H. (1991). Simian virus 40 Vp2/3 small structural proteins harbour their own nuclear transport signal. *Virology* 181: 78-90.

Emini, E.A., Hughes, J.V., Perlow, D.S. and Boger, J. (1985). Induction of hepatitis A virus-neutralizing antibody by a virus specific synthetic peptide. *Journal of Virology* 55: 836-839.

Flipsen, J.T.M., Van Lent, J.W.M., Goldbach, R.W. and Vlak, J.M. (1992). Expression of polyhedrin and p10 in the midgut of AcMNPV infected *Spodoptera exigua* larvae: an immunoelectron microscopic investigation. *Journal of Invertebrate Pathology* 61: 17-23.

Francki, R.I.B., Fauquet, C.M., Knudson, D. and Brown, F. (eds) (1991). Classification and nomenclature of viruses. Fifth Report of the International Committee on Taxonomy of Viruses. *Archives of Virology* supplementum II: pp 450.

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

Groebe, D.R., Chung, A.E. and Ho, C. (1990). Cationic lipid-mediated co-transfection of insect cells. *Nucleic Acids Research* 18: 4033.

Hink, F. (1970). Established insect cell line from the cabbage looper, *Trichoplusia ni*. *Nature (London)* 226: 446-467.

Jarvis, D.L., Bohlmeyer, D.A. and Garcia, Jr. A. (1991). Requirements for nuclear localization and supramolecular assembly of a baculovirus polyhedrin protein. *Virology* 185: 795-818.

Kuzio, J., Rohel, D.Z., Curry, C.J., Krebs, A., Carstens, E.H. and Faulkner, P. (1984). Nucleotide sequence of the p10 polypeptide gene of *Autographa californica* nuclear polyhedrosis virus. *Virology* 139: 414-418.

Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydrophobic character of a protein. *Journal of Molecular Biology* 157: 105-132.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227: 680-685.

Leisy, D.J., Rohrmann, G.F., Nesson, M. and Beaudreau, G.S. (1986). Nucleotide sequencing and transcriptional mapping of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus p10 gene. *Virology* 163: 157-167.

Mackinnon, E.A., Henderson, J.F., Stolz, D.B. and Faulkner, P. (1973). Morphogenesis of nuclear polyhedrosis virus under conditions of prolonged passage *in vitro*. *Journal of Ultrastructural Research* 49: 419-435.

Pugsley, A.P. (1989). The targeting of nuclear proteins. In *Protein targeting*, pp 199-210. San Diego: Academic Press.

Quant-Russell, R.L., Pearson, M.N., Rohrmann, G.F. and Beaudreau, G.S. (1987). Characterization of baculovirus p10 synthesis using monoclonal antibodies. *Virology* 160: 9-19.

Quant-Russell, R.L. and Rohrmann, G.F. (1990). A baculovirus polyhedron envelope protein: Immunogold localization in infected cells and mature polyhedra. *Virology* 174: 177-184.

Quant-Russell, R.L., Pearson, M.N. and Rohrmann, G.F. (1991). Immunoelectron microsopic examination of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus-infected *Lymantria dispar* cells: time course and localization of major polyhedron-associated proteins. *Journal of General Virology* 72: 275-283.

Rankin, C., Ladin, B.F. and Weaver, R.F. (1986). Physical mapping of temporally regulated overlapping transcripts in the region of the 10K protein of *Autographa californica* nuclear polyhedrosis virus. *Journal of Virology* 57: 18-27.

Roelvink, P.W., Van Meer, M.M.M., De Kort, C.A.D., Possee, R.D., Hammock, B.D. and Vlak, J.M. (1992). Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and p10 genes. *Journal of General Virology* 73: 1481-1489.

Rohel, D.Z., Cochran, M.A. and Faulkner, P. (1983). Characterization of two abundant mRNAs of *Autographa californica* nuclear polyhedrosis virus present late in infection. *Virology* 124: 357-365.

Rohrmann, G.F. (1987). Polyhedrine structure. *Journal of General Virology* 67: 1499-1513.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press.

Smith, G.E. and Summers, M.D. (1978). Analysis of baculovirus genomes with restriction enzyme endonucleases. *Virology* 98: 517-527.

Smith, G.E., Vlak, J.M. and Summers, M.D. (1982). *In vitro* translation of *Autographa californica* nuclear polyhedrosis virus early and late mRNAs. *Journal of Virology* 44: 199-208.

Smith, G.E., Vlak, J.M. and Summers, M.D. (1983). Physical analysis of *Autographa californica* nuclear polyhedrosis virus transcripts for polyhedrin and 10,000-molecular-weight protein. *Journal of Virology* 45: 215-225.

Van der Wilk, F., Van Lent, J.W.M. and Vlak, J.M. (1987). Immunogold detection of polyhedrin, p10 and virion antigens in *Autographa californica* nuclear polyhedrosis virus-infected *S. frugiperda* cells. *Journal of General Virology* 68: 2615-2623.

Van Lent, J.W.M., Groenen, J.T.M., Klinge-Roode, E.C., Rohrmann, G.F., Zuidema, D. and 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., Kormelink, R.J.M., Van Lent, J.W.M., Zuidema, D. and Vlak, J.M. (1990). Functional studies on the p10 protein of *Autographa californica* nuclear polyhedrosis virus. *Abstracts, VIIIth International Congress of Virology*, P4-007.

Van Strien, E.A., Zuidema, D., Goldbach, R.W. and 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. and McCawley, P. (1977). The establishment of two cell lines from the insect *Spodoptera frugiperda* (*Lepidoptera: Noctuidae*). *In Vitro* 13: 213-217.

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klinge-roode, E.C., Geervliet, J.B.F., Roosien, J. and Van Lent, J.W.M. (1988). Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-ß-galactosidase fusion gene. *Journal of General Virology* 69: 765-776.

Vlak, J.M., Schouten, A., Usmany, M., Belsham, G.J., Klinge-Roode, E.C., Maule, A.J., Van Lent, J.W.M. and Zuidema, D. (1990). Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. *Virology* 179: 312-320.

Volkman, L.E. and Zaal, K.J.M. (1990). *Autographa californica* M nuclear polyhedrosis virus: microtubules and replication. *Virology* 175: 292-302.

Whitt, M.A. and 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.

Williams, G.V., Rohel, D.Z., Kuzio, J. and Faulkner, P. (1989). A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *Journal of General Virology* 70: 187-202.

Wu, J., Daugulis, A.J., Faulkner, P. and Goosen, M.F.A (1992). Correlation of LDH activity with loss of insect cell viability: an assessment of LDH assay. *Biotechnology Techniques* 6: 335-340.

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

SPECIFICITY OF BACULOVIRUS P10 FUNCTIONS

SUMMARY

Three functional domains in baculovirus p10 proteins have been postulated for aggregation, nuclear disintegration and fibrillar structure formation (Van Oers *et al.*, *J. Gen. Virol.* 74: 563-574, 1993). To study the specificity of these functions, a recombinant *Autographa californica* nuclear polyhedrosis virus (AcCR1) was constructed in which the coding sequence of the p10 gene was replaced with the p10 sequence of the distantly related *Spodoptera exigua* (Se) MNPV. In AcCR1-infected cells the SeMNPV p10 protein was produced at similarly high levels as AcMNPV p10 in wild type (wt) AcMNPV infections. Formation of fibrillar structures occurred in a similar fashion in SeMNPV and AcCR1-infected cells. Hence, the SeMNPV p10 protein retained the ability to associate into fibrillar structures when expressed in an otherwise AcMNPV context. Mixed infection with wt AcMNPV and AcCR1 indicated that only p10 proteins of the same species aggregate and that these aggregates associate into fibrillar structures. In contrast to *S. exigua* cells infected with AcMNPV or SeMNPV, *S. exigua* cells infected with AcCR1 failed to release polyhedra. This result indicated that interaction of p10 with at least one virus-specific factor is required for nuclear disintegration.

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INTRODUCTION

Nuclear polyhedrosis viruses (NPVs), belonging to the family of *Baculoviridae* (Francki *et al.*, 1991), encode a small protein of approximately 10 kDa, that is expressed in large quantities late in infection of insect cells. This protein, denoted p10, is associated with fibrillar structures, which are found in both nucleus and cytoplasm of infected cells (Van der Wilk *et al.*, 1987; Quant-Russell *et al.*, 1987), and p10 has been suggested to be involved in the release of polyhedra from infected cells (Williams *et al.*, 1989). The DNA sequence of the p10 genes of six different NPVs has been reported (Kuzio *et al.*, 1984; Leisy *et al.*, 1986; Yaozhou, 1992; Chou *et al.*, 1992; Wilson *et al.*, 1992; Zuidema *et al.*, 1993). Although the predicted p10 proteins show a high divergence in amino acid sequence — the p10 proteins of *Autographa californica* (Ac) MNPV and *Spodoptera exigua* (Se) MNPV, for instance, have a sequence similarity of only 44% (Zuidema *et al.*, 1993) — their size, hydrophilicity distribution, and possibly secondary structure appeared to be conserved (Zuidema *et al.*, 1993).

In the p10 protein of AcMNPV three distinct functional domains have been postulated upon analysis of fusion and deletion mutants. For the amino-terminal domain an aggregation or polymerization function was suggested (Vlak *et al.*, 1988; Van Oers *et al.*, 1993). The hydrophilic, positively charged carboxy terminus is involved in the formation of the fibrillar structures (Van Oers *et al.*, 1993), possibly through interaction of p10 aggregates with tubulin (Volkman and Zaal, 1990; Cheley *et al.*, 1992). Such a positively charged carboxy terminus appears to be characteristic for baculovirus p10 proteins (Zuidema *et al.*, 1993). A central domain may be involved in nuclear disintegration, which leads to the release of polyhedra from infected cells (Van Oers *et al.*, 1993).

Due to the high degree of sequence variation among p10 proteins, questions were raised concerning the specificity of these functions. For example, is aggregation only possible between identical p10 proteins or can heteroaggregation occur between p10 proteins of different NPV species? Is the association of p10 aggregates into fibrillar structures the consequence of a specific interaction with the host? And also, is nuclear disintegration based on the interaction of p10 with virus- or host-specific factors? In order to answer these questions an AcMNPV recombinant has been constructed, in which the open reading frame of the authentic p10 gene is replaced by the SeMNPV p10 coding sequence. The cytopathological properties of this recombinant have been analysed.

MATERIALS AND METHODS

Cell culture, insect rearing, and virus isolates

The *Spodoptera frugiperda* cell line IPLB-SF-21 (Vaughn *et al.*, 1977) and the *S. exigua* cell line UCR-SE-1 (Gelernter and Federici, 1986b) were grown in plastic culture flasks in TNM-FH medium (Hink, 1970) supplemented with 10% (v/v) fetal calf serum. Larvae of *S. exigua* were taken from a culture that was maintained on an artificial diet at 25 ± 2°C, 70-80% relative humidity and a 16 h light/8 h dark photoperiod (Smits and Vlak, 1988).

The AcMNPV strain E2 (Smith and Summers, 1978) was used as wild type (wt) virus. A field isolate of SeMNPV (Gelernter and Federici, 1986a) was kindly provided by Dr B.A. Federici. The AcMNPV recombinant AcMO16 (Van Oers *et al.*, 1993) was used as a positive control and contained a full-length copy of the AcMNPV p10 coding sequence in its original location (Fig. 5.1 B). The AcMNPV p10 deletion mutant AcAS3 (Vlak *et al.*, 1990) was used as a negative control for the expression of p10 (Fig. 5.1 B).

Construction of a p10 transfer vector and purification of a recombinant virus

The 5.1 kb SeMNPV *Xba*I-H fragment cloned into pTZ18R (Zuidema *et al.*, 1993) was chosen as source for the SeMNPV p10 coding sequence (Fig. 5.1 A). This plasmid was linearized with *Sca*I and used as template in a polymerase chain reaction (PCR). Using primers specific for the 5' and 3' ends, the SeMNPV p10 coding sequence was amplified from positions −3 to +267 relative to the ATG start codon. The amplified DNA segment included the entire p10 coding sequence, the three base pairs preceding the ATG start codon, and the translational stop codon. To facilitate cloning, *Bam*HI sites were located at both ends of the PCR product. Therefor, the primers contained additional, non-homologous sequences at their 5' ends: the 5' end-specific primer (5' GGGGATCCATTATGAGTCAAAATATTTTAC 3') was complementary to the anticoding strand from positions −3 to +19 and the 3' end-specific primer (5' GGGGATCCTTAC-TTTTTGTTGCTTTTCT 3') was complementary to the coding strand from nucleotide residue +267 to +248. The reaction conditions were as described previously (Van Oers *et al.*, 1993).

The PCR product was digested with *Bam*HI and inserted into pUC19 (pSeCR1). The integrity of the insert was checked by DNA sequencing using standard techniques. The sequenced PCR product was recloned into the *Bam*HI site of the AcMNPV p10 promoter-based transfer vector pAcAS3 (Vlak *et al.*, 1990) to give the transfer vector pAcCR1 (Fig. 5.1 B). The orientation of the SeMNPV p10 insert was confirmed by restriction enzyme analysis and by DNA sequencing using an AcMNPV p10 promoter-specific primer

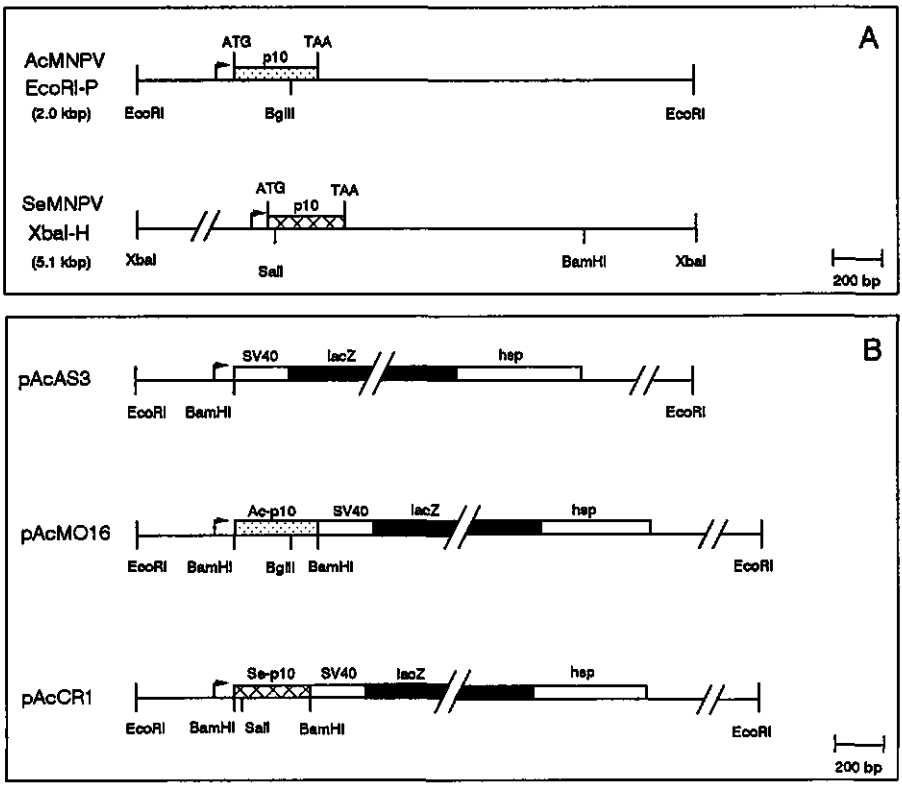

Figure 5.1. A) Schematic representation of the AcMNPV fragment *Eco*RI-P (Smith *et al.*, 1983) and *Se*MNPV fragment *Xba*I-H (Zuidema *et al.*, 1993) containing the respective p10 genes. B) Schematic representation of transfer vectors pAcAS3 (Vlak *et al.*, 1990) used as parental plasmid, and pAcMO16 (Van Oers *et al.*, 1993) containing the AcMNPV p10 coding sequence, and pAcCR1 with the SeMNPV p10 coding sequence. All transfer vectors contained a gene cassette containing the hsp70 promoter, the lacZ coding sequence and the SV40 terminator to allow recombinant virus selection. Arrows indicate the p10 mRNA start sites. Dotted boxes represent the AcMNPV and hatched boxes the SeMNPV p10 coding sequence. Only those restriction sites used in this study are indicated.

(5' GAATTATTATCAAATCATTTG 3'), complementary to the anticoding strand from positions −68 to −48, relative to the authentic AcMNPV p10 translational start codon. DNA sequencing revealed that a T at position +66 relative to the ATG start codon of the SeMNPV p10 gene, was substituted by an A, but the encoded amino acid remained an alanine.

Recombinant baculovirus was produced by lipofectin-mediated cotransfection (Groebe *et al.*, 1990) of SF-21 cells with the transfer vector pAcCR1 and wt AcMNPV DNA. Recombinant virus (AcCR1) was selected as blue-coloured plaques after incubation with X-gal (Boehringer-Mannheim) and plaque purified by standard methods (Brown and Faulkner, 1977). Viral DNA was isolated from extra cellular viruses (ECVs) and analysed by digestion with *Bgl*II and *Hin*dIII. As a further control DNA sequencing was performed with the CircumVent thermal cycle dideoxy DNA sequencing kit (New England Biolabs) on 1 μg viral DNA isolated from ECVs and 2.5 pmol of the AcMNPV p10 promoter-specific primer mentioned above.

Production of antisera against the AcMNPV and SeMNPV p10 proteins

SF-21 cells were infected with either wt AcMNPV or with recombinant AcCR1 at a multiplicity of infection (m.o.i.) of 5 $TCID_{50}$ units/cell to produce AcMNPV and SeMNPV-specific p10. Cells were harvested at 48 h post infection (p.i.), washed twice in phosphate buffered saline (PBS), and resuspended in protein sample buffer (Laemmli, 1970). The protein sample was electrophoresed through 1.5 mm-thick preparative SDS-polyacrylamide gels with a polyacrylamide gradient from 10 to 18% (BIO-RAD Protein System II). The gels were superficially stained in Coomassie brilliant blue for 10 min and destained in 5% acetic acid, 7.5% methanol for 15 min. The p10 protein band was cut from the gels and the protein was eluted in a BIO-RAD electro-eluter (Model 422) in a partly volatile buffer (50 mM NH_4HCO_2, 0.1% SDS) as recommended by the manufacturer. The eluate obtained was freeze-dried overnight and subsequently dissolved in 40 mM Tris•HCl, pH 6.8; 4 mM EDTA, pH 8.0.

After obtaining preimmune sera, rabbits were injected subcutaneously with approximately 30 μg of p10 protein, which was mixed 1:1 with incomplete Freund's adjuvant. A booster injection was given after 3 weeks with approximately 70 μg of p10. For the AcMNPV p10 antiserum another two boosters were given with approximately 50 μg of p10 with intervals of 3 weeks. After the last booster serum was collected three times over a period of 10 weeks.

Protein analysis

SF-21 and UCR-SE-1 cells were infected with wt AcMNPV, AcMO16, AcCR1, and AcAS3 at a m.o.i. of 10 $TCID_{50}$ units/cell. UCR-SE-1 cells were infected with SeMNPV at 1 $TCID_{50}$ unit/cell. This difference in m.o.i. was due to practical considerations, since SeMNPV-infected cells produce less budded virions than AcMNPV infected cells. Cells were harvested at 48 h p.i. and the protein patterns were analysed by SDS-PAGE according to Laemmli (1970) in 13.5% polyacrylamide gels (BIO-RAD Mini-Protean^{II} apparatus). The gels were stained with Coomassie brilliant blue or blotted onto nitrocellulose. Western blot analysis was performed as described previously (Van Oers *et al.*, 1993). Antiserum raised against the AcMNPV p10 protein was diluted 1:1000 and antiserum against the SeMNPV p10 protein 1:3000. Goat-anti-rabbit immunoglobulins conjugated to alkaline phosphatase (Tago, Burlingham, CA) were diluted 1:1000.

Electron microscopy and immunogold labelling

SF-21 and UCR-SE-1 cells were infected at a m.o.i. of 10 $TCID_{50}$ units/cell with wt AcMNPV, AcMO16, AcAS3, and AcCR1 and harvested at 46 h p.i. UCR-SE-1 cells were infected with SeMNPV at a m.o.i. of 3 $TCID_{50}$ unit/cell, and harvested at 48 h p.i. Cells were embedded in LR Gold resin (London Resin Company) as described by Van Lent *et al.* (1990) with some modifications (Van Oers *et al.*, 1993). Ultrathin sections were cut with a diamond knife on a Reichert-Jung Ultracut^{E} microtome and mounted on nickel grids coated with a formvar supporting film. Immunogold labelling was performed as described by Van Lent *et al.* (1990) with 1:200 diluted AcMNPV p10 antiserum or 1:1000 diluted SeMNPV p10 antiserum, and subsequently with Protein A-gold with a diameter of 7 nm. Immunogold labelled sections were stained as described by Flipsen *et al.*, (1993) before examination in a Philips CM12 transmission electron microscope.

Double-infection experiments

SF-21 cells were double-infected with AcCR1 and wt AcMNPV (both at a m.o.i. of 10 $TCID_{50}$ units/cell) and harvested at 48 h p.i. The protein pattern was analysed and compared to single infections with either wt AcMNPV or AcCR1 (m.o.i. 20) in 13.5% SDS-PAGE. P10 proteins were detected by Western blot analysis (as described above). Viral DNA was isolated at 48 h p.i. from progeny ECVs and analysed by digestion with *Bgl*II and electrophoresis through a 0.4% agarose gel.

Double-infected SF-21 and UCR-SE-1 cells were also processed for electron microscopy and serial ultrathin sections were mounted on slot grids with a formvar supporting film. Immunogold labelling of p10 proteins was performed as described above.

Release of polyhedra

SF-21 and UCR-SE-1 cells were infected at a m.o.i. of 10 $TCID_{50}$ units/cell with wt AcMNPV, AcAS3, AcMO16 and AcCR1. The infected-cell cultures were examined at 5 and 8 days p.i. by phase-contrast microscopy with a Leitz Labovert FS microscope and photographs were taken. UCR-SE-1 cells infected with SeMNPV at a m.o.i. of 1 $TCID_{50}$ unit/cell served as positive controls for release of polyhedra induced by the SeMNPV p10 protein.

Infection of larvae

Infected SF-21 cells were harvested at 72 h p.i., washed twice in PBS and incubated in 0.5% Nonidet in PBS at room temperature for 10 min. The disrupted cells were washed twice in PBS, resuspended in PBS under sonication (Type B12, Branson Sonic Power Company, CT, USA) and stored at −20°C until further use. Fourth instar *Spodoptera exigua* larvae, starved overnight, were placed separately in wells of 24-well tissue culture plates (Costar). Larvae were infected with either wt AcMNPV, AcAS3, AcMO16 or AcCR1 at a dose of 1 x 10^6 polyhedra per larva. The polyhedra were supplied on a small piece of diet, which was replaced by new diet 48 h later. Control larvae were fed on diet only. The mortality was scored 5 days after infection.

RESULTS

Construction of an AcMNPV recombinant encoding the SeMNPV p10 protein

To examine the influence of host cell and parental virus proteins on the function of p10, the p10 coding sequence in AcMNPV was replaced with the distantly related p10 sequence of SeMNPV. For these investigations a number of viruses were used including SeMNPV, the AcMNPV p10 deletion mutant AcAS3 (Vlak *et al.*, 1990), AcMNPV recombinant AcMO16 (Van Oers *et al.*, 1993) encoding a wt AcMNPV p10 protein, and AcMNPV recombinant AcCR1, in which the coding sequence of the authentic p10 gene was replaced with the SeMNPV p10 coding sequence (see Material and Methods). The recombinant virus AcCR1 was constructed with the AcMNPV p10 promoter to minimize regulatory changes.

Expression and identification of AcMNPV and SeMNPV p10 proteins

The expression of p10 protein by wt AcMNPV and recombinant viruses was analysed in SF-21 (Fig. 5.2 A) and UCR-SE-1 (Fig. 5.2 B) cells, whereas SeMNPV was tested on UCR-SE-1 cells. SeMNPV only replicates efficiently in UCR-SE-1 cells. SF-21 and UCR-SE-1 cells infected with the positive control AcMO16 (Fig. 5.2 A and 5.2 B;

lanes 4) showed a p10 of the same size as wt AcMNPV-infected cells (lanes 2), as has been observed previously for SF-21 cells (Van Oers *et al.*, 1993). As expected, p10 protein was absent from AcAS3-infected cells (Fig. 5.2 A and 5.2 B; lanes 3). Cells infected with recombinant AcCR1 (Figs. 5.2 A and 5.2 B; lanes 5) produced a p10 protein that was slightly smaller than the AcMO16 p10 protein (lanes 4), and that comigrated with the SeMNPV p10 protein (Fig. 5.2 B; lane 6). The level of p10 production in both SF-21 and UCR-SE-1 cells was comparable. This analysis confirmed

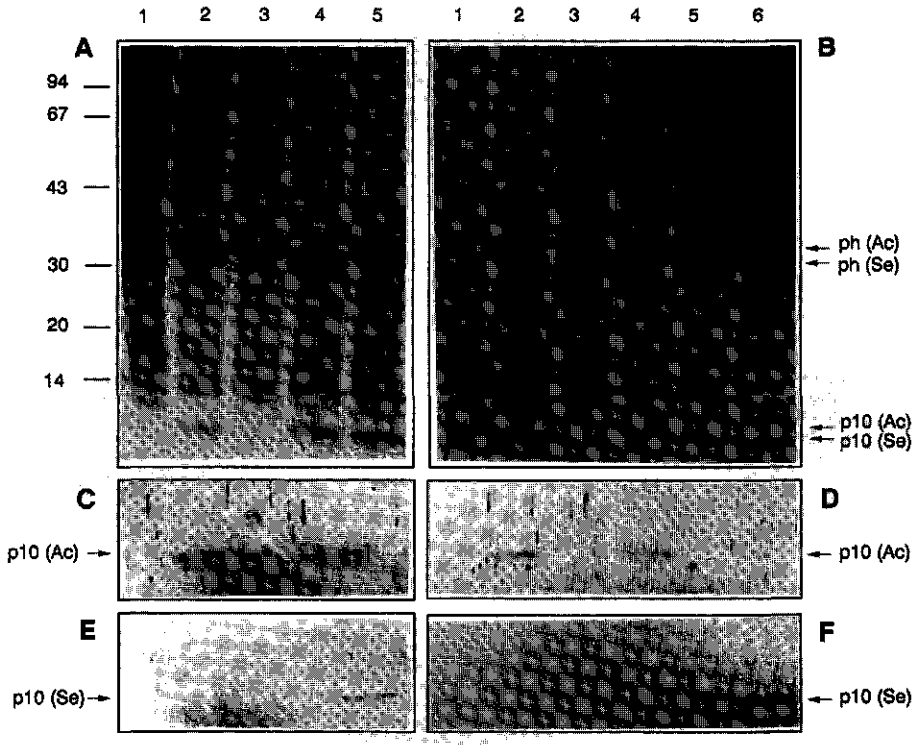

Figure 5.2. A) SF-21 cells mock infected (lane 1) or infected with wt AcMNPV (lane 2), AcAS3 (lane 3), AcMO16 (lane 4) and AcCR1 (lane 5) were harvested at 48 h p.i. and analysed in a 13.5% SDS-polyacrylamide gel. B) UCR-SE-1 cells were infected and analysed as described for (A) in addition to UCR-SE-1 cells infected with SeMNPV (lane 6). AcMNPV p10 antiserum was used to detect AcMNPV p10 protein on Western blots of infected SF-21 (C) and UCR-SE-1 cells (D). A sister blot was treated with SeMNPV p10 antiserum (E) and (F). Each lane contained the protein equivalent of 4 a 10^4 cells. Molecular weight markers are indicated in 10^3 Da. Arrows indicated the positions of polyhedrin and p10 proteins.

confirmed that the size of SeMNPV polyhedrin (30 kDa) is smaller than that of the AcMNPV polyhedrin (33 kDa) (Caballero *et al.*, 1992). Furthermore, there was no difference observed in AcMNPV polyhedrin production between the two cell lines.

To identify and distinguish the p10 proteins of AcMNPV and SeMNPV, antisera were raised against the p10 proteins produced in wt AcMNPV and AcCR1-infected SF-21 cells. The specificity of the antisera was tested by Western blot analysis on infected-cell extracts of both cell lines (Fig. 5.2 C-F). The AcMNPV p10 antiserum (Fig. 5.2 C and D) reacted only with the p10 protein in AcMNPV (lanes 2) and AcMO16-infected cells (lanes 4). Proteins of mock (lanes 1) or AcAS3-infected cells (lanes 3) did not react specificially with either antiserum. The AcMNPV p10 antiserum did not cross-react with the p10 protein of AcCR1 (Fig. 5.2 C and D; lanes 5) or of SeMNPV (Fig. 5.2 D; lane 6). The antiserum raised against the p10 protein of AcCR1 was specific for the p10 protein in AcCR1 (Fig. 5.2 E and F; lanes 5) and SeMNPV-infected cells (Fig. 5.2 F; lane 6). No reactions were observed when the preimmune sera were used (data not shown).

Fibrillar structures in AcMNPV and SeMNPV infected UCR-SE-1 cells

Ultrathin sections of UCR-SE-1 cells infected with wt AcMNPV or SeMNPV were examined for the formation of fibrillar structures by immunogold labelling with the p10 specific antisera. With the AcMNPV p10 antiserum only fibrillar structures in AcMNPV-infected cells were labelled (Fig. 5.3 A), whereas no cross-reaction with other structures was observed. The SeMNPV p10 serum was specific for SeMNPV-infected cells, where it also detected fibrillar structures in the nucleus and cytoplasm (Fig. 5.3 B). These fibrillar structures were more dense than those induced by AcMNPV. Preimmune sera did not react with any infected-cell structures and the p10 antisera did not show mutual cross-reactivity (data not shown). These results indicate that, like the AcMNPV p10 protein (Fig. 5.3 A), the p10 protein of SeMNPV was associated with fibrillar structures present both in nuclei and cytoplasm of infected UCR-SE-1 cells (Fig. 5.3 B), but that there is a difference in the fibrils formed by these proteins. In the nucleus the fibrillar structures of AcMNPV and SeMNPV were often associated with electron dense spacers.

Cytopathology of the recombinant AcCR1

To study whether the SeMNPV p10 protein, when expressed in an AcMNPV context, SF-21 able to associate into fibrillar structures, AcCR1 and AcMO16-infected SF-21 and UCR-SE-1 cells were collected at 46 h p.i. and processed for electron microscopy.As expected, cells infected with the control AcMO16 (Fig. 5.3 C) produced fibrillar structures, which contained the AcMNPV p10 protein and which resembled the fibrillar structures obtained in a wt AcMNPV infection (Fig. 5.3 A). The p10 protein in AcCR1-

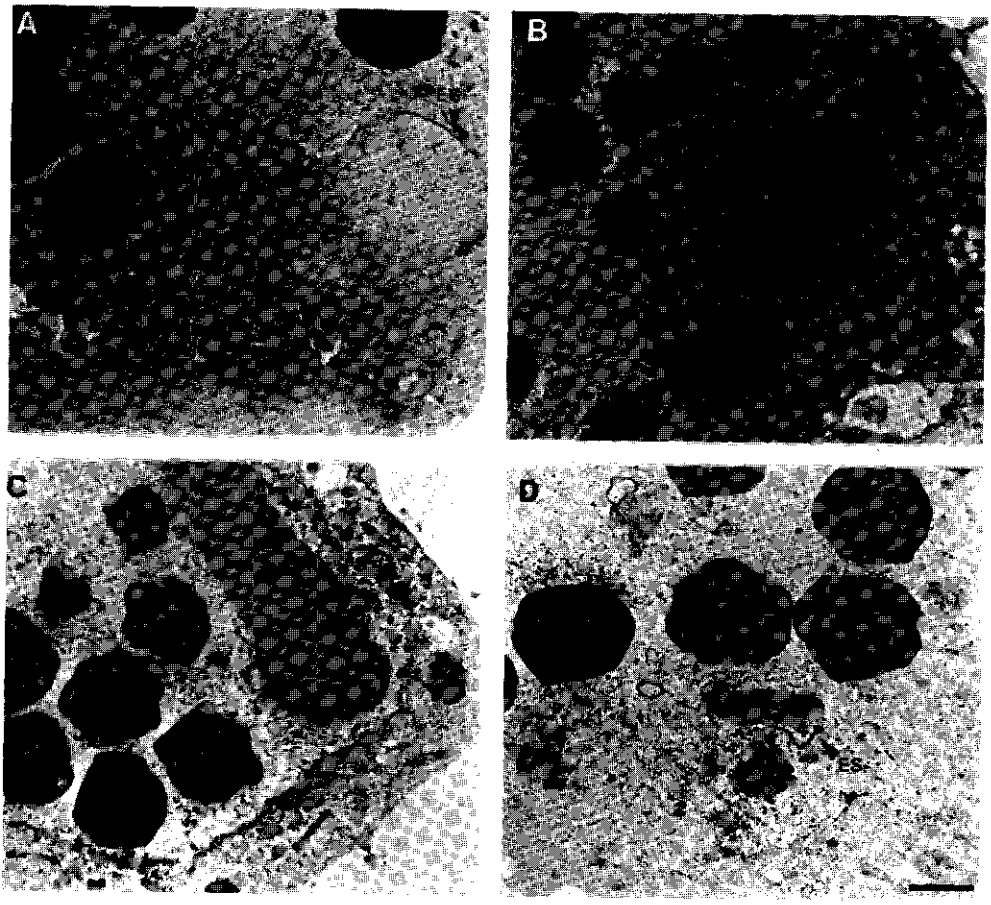

Figure 5.3. Electron micrographs of immunogold labelled thin sections of UCR-SE-1 cells at 46 h p.i. with wt AcMNPV (A), 48 h p.i. with SeMNPV (B) and of SF-21 cells at 46 h p.i. with AcMO16 (C) or AcCR1 (D). Sections of wt AcMNPV- or AcMO16-infected cells were treated with AcMNPV p10 antiserum (A and C). Sections of SeMNPV- and AcCR1-infected cells were incubated with SeMNPV p10 antiserum (B and D). Subsequently, all sections were incubated with Protein A-gold. Fibrillar structures (F) and electron-dense spacers (ES) are indicated. The bar marker represents 1 μm.

infected SF-21 cells was detected with the SeMNPV p10 antiserum and was also present in fibrillar structures (Fig. 5.3 D), that were formed in both the nucleus and the cytoplasm. These fibrillar structures had the same dense appearance as the fibrillar structures in SeMNPV-infected cells. Similar results were obtained for both AcMO16 and AcCR1 when UCR-SE-1 cells were used (data not shown). The association of fibrillar structures with electron dense spacers appeared to be less intimate in AcCR1-infected cells than in wt AcMNPV- or SeMNPV-infected cells. The polyhedra, produced by AcCR1, were surrounded by envelopes as wt AcMNPV and SeMNPV polyhedra.

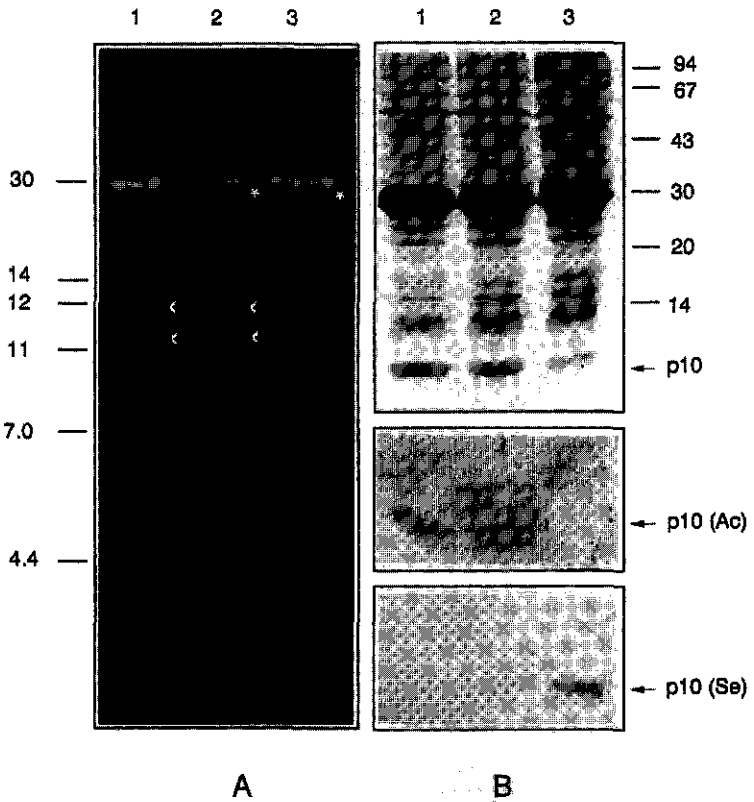

Figure 5.4. SF-21 cells were infected with wt AcMNPV (lanes 1), with AcCR1 (lanes 3) or simultaneously with wt AcMNPV and AcCR1 (lanes 2). A) DNA was extracted from nonoccluded virions at 48 h p.i. and the *Bgl*II restriction enzyme pattern was analysed in a 0.4% agarose gel. Arrows indicate an 11.8 kbp and an 11.3 *Bgl*II fragment characteristic for wt AcMNPV, asterisks indicate a 26.8 kbp fragment characteristic for AcCR1. B) The infected cells were harvested at 48 h p.i. and analysed in a 13.5% SDS-polyacrylamide gel. Following SDS-PAGE the proteins were transferred to nitrocellulose and Western blot analysis was performed with antiserum against AcMNPV (upper blot) or SeMNPV (lower blot) p10 protein.

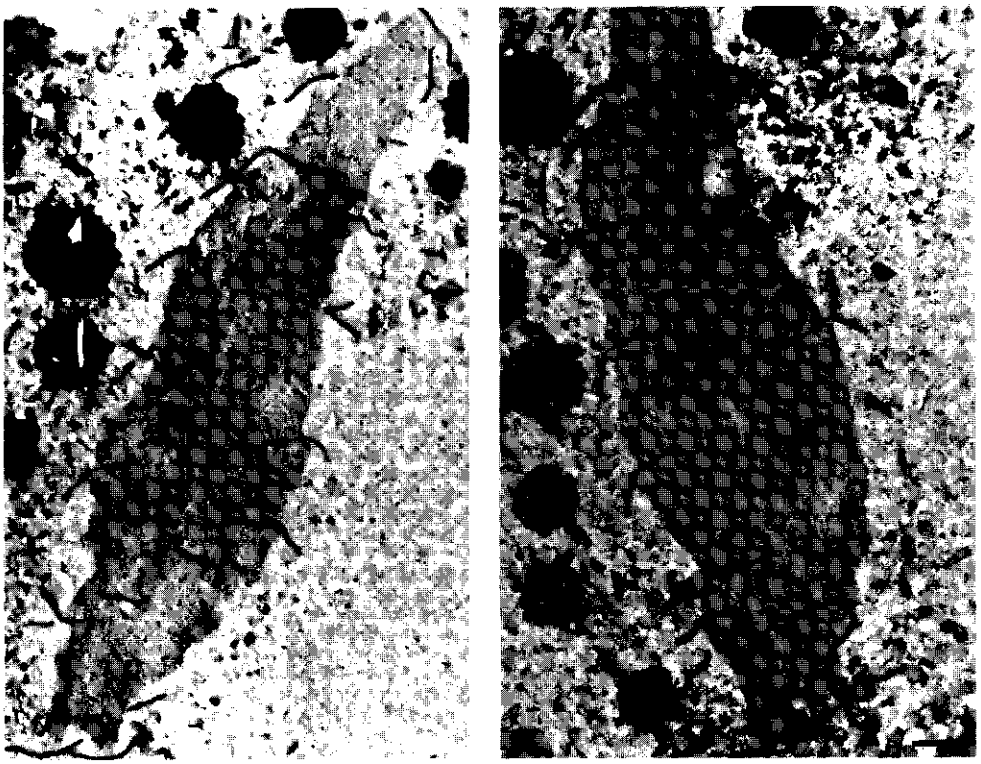

Figure 5.5. Fibrillar structure in SF-21 cells co-infected with wt AcMNPV and AcCR1 at 46 h p.i. shown in two serial ultrathin sections of which (A) is incubated with AcMNPV p10 antiserum and (B) with SeMNPV p10 antiserum. Both sections were subsequently complexed with Protein A-gold. The bar marker represents 0.5 μm.

Coexpression of AcMNPV and SeMNPV p10 proteins

To examine whether heteropolymerization occurred between the AcMNPV and SeMNPV p10 proteins, SF-21 cells were co-infected with wt AcMNPV and AcCR1 both at a m.o.i. of 10 $TCID_{50}$ units/cell and examined 48 h after infection. The DNA pattern of the progeny virus was analysed upon digestion with *Bgl*II (Fig. 5.4 A). With the exchange of p10 sequences the *Bgl*II site, indicative for the AcMNPV p10 gene, has been lost (see Fig. 5.1). This resulted in a difference in the *Bgl*II digestion pattern between wt AcMNPV and AcCR1. *Bgl*II fragments of 11.8 and 11.3 kbp, present in wt AcMNPV DNA (Fig. 5.4 A; lane 1, note arrows), were absent from AcCR1 DNA (Fig. 5.4 A; lane 3). Instead, a new band of 26.8 kbp was observed in AcCR1 DNA (lane 3; note asterisk), which corresponded to the total size of the 11.8 and 11.3 kbp genomic

fragments and a 3.8 kbp fragment derived from the HSP-lacZ-SV40 gene cassette. In the DNA pattern of ECVs produced by co-infected cells (Fig. 5.4 A; lane 2) *Bgl*II fragments characteristic for both wt AcMNPV (arrows) and AcCR1 (asterisk) were represented. The 11.8, 11.3 and 26.8 kbp fragments were present in appproximately half-molar amounts, in accordance with the fact that only half of the progeny virus genomes contained these fragments. The results indicate that both wt AcMNPV and AcCR1 were replicating and that the co-infection with these two viruses had occurred at similar m.o.i.'s. The protein pattern of mix-infected cells showed that both p10 proteins were expressed as can be seen most clearly upon Western blot analysis (Fig. 5.4 B; lane 2). To determine whether the two p10 proteins coaggregated in these mix-infected cells, serial ultrathin sections were mounted on different slot grids and labelled with antiserum for either AcMNPV or SeMNPV p10. Electromicrographs revealed that both p10 proteins were present in the same fibrillar structure, but were not homogeneously distributed (Fig. 5.5 A and B). The fibrillar structures contained confined areas of fibrils with either AcMNPV or SeMNPV p10 protein. The SeMNPV p10 containing fibrils had a more condensed appearance. The amount of AcMNPV p10 and SeMNPV p10 in a fibrillar structure varied per cell, probably due to differences in the entry ratio of wt versus recombinant virus. Cells with only AcMNPV or SeMNPV p10 were also observed.

Release of polyhedra

Since p10 is involved in the disintegration of infected cell nuclei, UCR-SE-1 cells were infected with wt AcMNPV, AcMO16, AcAS3, AcCR1, and SeMNPV and analysed for the release of polyhedra by phase contrast microscopy 5 days after infection (Fig. 5.6). Many free polyhedra were observed in cell cultures infected with AcMO16 (Fig. 5.6 A) and wt AcMNPV (not shown) as has been observed previously for SF-21 cells (Van Oers *et al.*, 1993). Extensive liberation of polyhedra was also observed upon infection of UCR-SE-1 cells with SeMNPV (Fig. 5.6 B). In contrast, in cell cultures infected with AcCR1 only occasionally a free polyhedron was found (Fig. 5.6 C) and this was still the case at 8 days p.i. This suggests that only a very small percentage of the nuclei had disintegrated. Similar results were obtained for SF-21 cells infected with AcCR1. The inability of AcCR1 to release polyhedra was not due to a possible mutation in the AcCR1 p10 sequence, since a correct p10 gene was found upon DNA sequencing of the viral DNA, and since a second, independently isolated AcCR1 recombinant gave the same results. UCR-SE-1 cells, infected with the p10 deletion mutant AcAS3 (Fig. 5.6 D), were completely impaired in the release of polyhedra, as has been observed previously for SF-21 cells (Van Oers *et al.*, 1993).

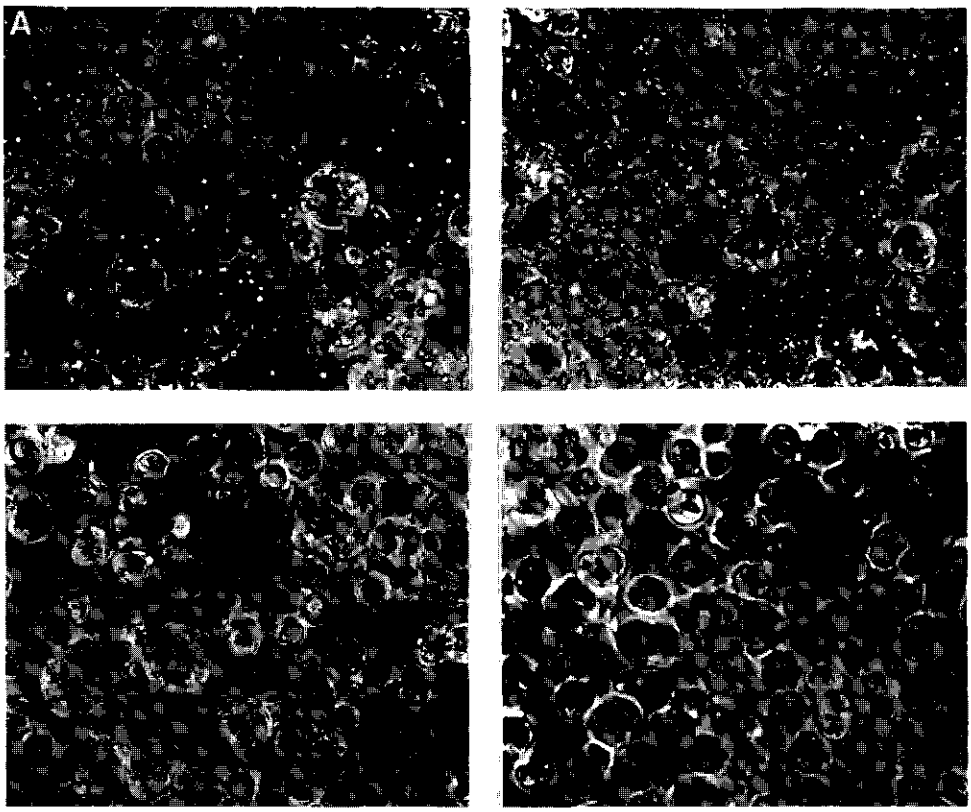

Figure 5.6. Phase-contrast micrographs of UCR-SE-1 cell cultures infected with AcMO16 (A), SeMNPV (B), AcCR1 (C) and AcAS3 (D) at 5 days after infection. The bar marker represents 20 μm.

Infectivity of the recombinant AcCR1

Normal nonoccluded virus titers (10^8-10^9 $TCID_{50}$ units/ml) were easily obtained with AcCR1, indicating that their production in cell cultures was comparable to that of wt AcMNPV. To investigate whether the heterologous expression of the SeMNPV p10 protein changed the infectivity of AcCR1 for larvae, *S. exigua* larvae were fed on diet containing AcCR1 polyhedra. The recombinant AcCR1 was able to cause a lethal infection, as were wt AcMNPV, the positive control AcMO16 and the p10 deletion mutant AcAS3. All infected larvae liquefied upon death, irrespective of whether wild type or recombinant viruses were used.

Table 1. Amino acid sequence similarity and identity between baculovirus p10 proteins[1]

	BmNPV	CfMNPV	OpMNPV	PnMNPV	SeMNPV
AcMNPV	91.5 (87.2)	66.7 (51.9)	61.5 (41.8)	62.6 (42.9)	44.3 (21.6)
BmNPV	—	65.0 (48.8)	61.5 (39.6)	61.5 (40.7)	51.2 (28.6)
CfMNPV	—	—	63.0 (50.6)	63.0 (45.7)	55.0 (37.5)
OpMNPV	—	—	—	98.9 (94.6)	55.4 (39.2)
PnMNPV	—	—	—	—	58.1 (39.2)

[1] The amino acid sequence similarity (%) and identity (%) were calculated with the UWGCG computer software package with a gap weight of 3.00 and a gap length weight of 0.10. The p10 sequences were retrieved from literature or extracted from the EMBL-BANK: AcMNPV p10 from Kuzio *et al.* (1984), *Bombyx mori* (Bm) NPV p10 from Yaozhou (1992), *Choristoneura fumiferana* (Cf) MNPV p10 from Wilson *et al.* (1992), *Orgyia pseudotsugata* (Op) MNPV p10 from Leisy *et al.* (1986), *Perina nuda* (Pn) MNPV from Chou *et al.* (1992) and SeMNPV p10 protein from Zuidema *et al.* (1993).

DISCUSSION

Late in infection nuclear polyhedrosis viruses express two proteins at very high levels: polyhedrin, the major component of polyhedra, and p10, which is involved in the formation of fibrillar structures and in the release of polyhedra. The amino acid sequence of polyhedrin is highly conserved (Rohrmann, 1986), whereas the sequence of p10 is less conserved (Fig. 5.7; Table 5.1). The highest variability is observed between the p10 proteins of AcMNPV and SeMNPV, which exhibit only 44.3% similarity and 21.6% identity (Zuidema *et al.*, 1993). Despite of this, the overall structure, including size and hydrophilicity, of p10 appear to be preserved, suggesting common functions. Based on deletion analysis a model in which both the amino and carboxy terminus are involved in fibrillar structure formation was proposed; the amino terminus in p10 aggregation and the carboxy terminus in the alignment of p10 aggregates in cooperation with the host (Vlak *et al.*, 1988; Van Oers *et al.*, 1993). A more central domain would be responsible for nuclear disintegration, leading to the release of polyhedra (Williams *et al.*, 1989; Van Oers *et al.*, 1993). In this study we have investigated whether p10 proteins, despite their interviral rate of variation, can be functionally interchanged between NPVs. To this end an AcMNPV recombinant (AcCR1) was constructed and analysed, in which the authentic p10 coding sequence was replaced with the coding sequence of SeMNPV p10. The

AcMNPV p10 promoter in its natural locus was chosen to drive p10 gene expression in order to minimally disturb the regulation by AcMNPV-encoded factors. In cells infected with AcCR1, p10 polypeptides were present in fibrillar structures in the nucleus and cytoplasm (Fig. 5.3 D). Hence, the SeMNPV p10 protein retained the ability to associate into these structures when expressed in an AcMNPV context. Previous deletion studies revealed that the eight carboxy-terminal amino acids (RRGKRSSK) of AcMNPV p10 are necessary for the arrangement of aggregated p10 into fibrillar structures and it was postulated that this domain interacts with tubulin (Van Oers *et al.*, 1993). All known p10 proteins have a carboxy-terminus with a hydrophilic and basic character, and contain at least one serine in this domain (Fig. 5.7; double underlined sequences). Cheley *et al.* (1992) showed that the AcMNPV p10 protein was converted into a heat-stable, microtubule-associated protein (MAP) upon artificial phosphorylation of one of the serine residues in this carboxy-terminal domain. Colocalization of p10 with microtubules was also observed and was most prominent in cytochalasin D (CD) treated cells (Volkman and Zaal, 1990). Stabilization of microtubules by artificially phosphorylated p10 or by CD apparently resulted in the formation of cell retraction processes in which microtubules were associated with p10. However, in wt AcMNPV infections microtubules depolymerize (Volkman and Zaal, 1990) and p10 polypeptides seem to be largely unphosphorylated (Cheley *et al.*, 1992). The fact that some MAPs also contain a basic sequence in their tubulin-binding domain (Goedert *et al.*, 1991) further supports the hypothesis that the basic carboxy terminus of p10 is involved in tubulin binding. Site-directed mutagenesis in the carboxy-terminal region of p10 will be performed to further analyse this putative interaction.

A monoclonal antibody against the *Orgyia pseudotsugata* (Op) MNPV p10 protein reacted also with microtubules in uninfected cells and the epitope was located in the sequence LPEIPDVP of p10 (Quant-Russell *et al.*, 1987). Such a sequence is not present in tubulin, but might occur in an associated protein as was suggested by these authors. Part of this sequence matches with an homology box found in all baculovirus p10 proteins (Zuidema *et al.*, 1993), which is characterized by the consensus sequence (A/V/L/I)P(D/E)(V/L/I/P)P (Fig. 5.7; underlined sequences). This may indicate that, in addition to the carboxy-terminus, another domain is involved in binding with tubulin.

In mixed-infections with wt AcMNPV and AcCR1 the fibrillar structures were assembled with confined areas, which contained either AcMNPV or SeMNPV p10 protein (Fig. 5.5 A and B). This distribution indicates that p10 proteins have a preference to self-aggregate. An aggregation function was predicted for the amino terminus of AcMNPV p10 (Vlak *et al.*, 1988). The amino termini of SeMNPV and AcMNPV p10 differ considerably in amino acid sequence, except for an homology box with the consensus

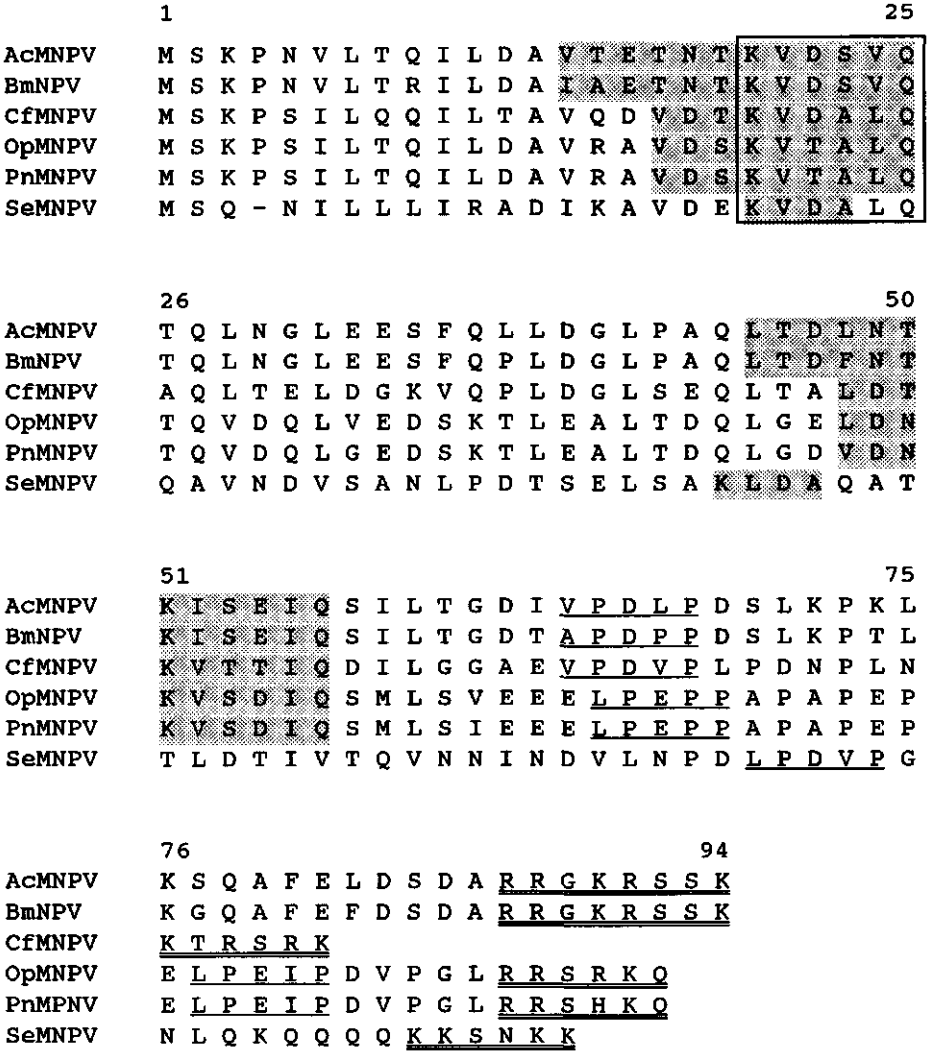

Figure 5.7. Amino acid sequence of p10 proteins as deduced from the DNA sequence of AcMNPV (Kuzio *et al.*, 1984), *Bombyx mori* (Bm) NPV (Yaozhou, 1992), *Choristoneura fumiferana* (Cf) MNPV (Wilson *et al.*, 1992), *Orgyia pseudotsugata* (Se) MPNV (Leisy *et al.*, 1986), *Perina nuda* (Pn) MNPV (Chou *et al.*, 1992) and SeMNPV (Zuidema *et al.*, 1993).

sequence KV(D/T)(A/S)(V/L)Q (Fig. 5.7; boxed). In fact, this box is part of a sequence with internal homology within the p10 protein. Such a repeat is found in all p10 molecules (Fig. 5.7; grey-shadowed characters). The function of this repeated sequence is not known, but it might be involved in aggregation or polymerization of p10 molecules. The alignment of homo-aggregates of p10 into fibrillar structures might be based on a cooperation of p10 with the host protein tubulin, which recognizes both AcMNPV and SeMNPV p10 aggregates.Homology box I is indicated as a box and has the consensus sequence KV(D/T)(A/S)(V/L)Q. A region with internal homology is grey-shadowed. Homology box II is characterized by the consensus (A/V/L/I)P(D/E)(V/L/I/P)P and is underlined. Note that this consensus sequence is present twice in the p10 proteins of OpMNPV and PnMNPV. The hydrophilic, positively-charged carboxy-termini are double underlined. Numbers indicate amino acid positions.

Cells infected with AcCR1 failed to release polyhedra late in infection in contrast to cells infected by AcMNPV, the control recombinant AcMO16 or SeMNPV (Fig. 5.6), and despite the formation of fibrillar structures. That nuclear disintegration and fibrillar structure formation are two separate functions of p10 has been shown previously for AcMNPV p10 (Van Oers *et al.*, 1993). Assuming that the mechanism of polyhedron release is identical for SeMNPV and AcMNPV, we can only explain the inability of AcCR1 to release polyhedra by postulating that one or more viral factors interact with p10 from the same virus species, and that this complex in turn induces nuclear disintegration and hence polyhedron release. Previously, we postulated (Van Oers *et al.*, 1993) that the nuclear disintegration function of AcMNPV p10 is located between amino acid residues 52 and 79. An AcMNPV recombinant expressing a chimeric p10 protein, containing the putative AcMNPV disintegration domain flanked by amino- and carboxy-terminal domains of SeMNPV p10 could be used to test this hypothesis. Once confirmed, further reducing the AcMNPV derived sequences might help to trace the site, which interacts with the proposed viral disintegration factor more precisely.

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REFERENCES

Brown, M. and Faulkner, P. (1977). A plaque assay for nuclear polyhedrosis viruses using a solid overlay. *Journal of General Virology* 36: 361-364.

Caballero, P., Zuidema, D., Santiago-Alvarez, C. and Vlak, J.M. (1992). Biochemical and biological characterization of four isolates of *Spodoptera exigua* nuclear polyhedrosis virus . *Biocontrol Science and Technology* 2: 145-157.

Cheley, S., Kosik, K.S., Paskevich, P., Bakalis, S. and Bayley, H. (1992). Phosphorylated baculovirus p10 is a heat-stable microtubule-associated protein associated with process formation in Sf-9 cells. *Journal of Cell Science* 102: 739-752.

Chou, J.M, Lo, C.F., Huang, C.J. and Wang, C.H. (1992). Isolation and nucleotide sequence of *perina nuda* multinucleocapsids nuclear polyhedrosis virus (pnMNPV) two late genes: Polyhedrin and p10 gene. *Proceedings of the XIX International Congress of Entomology, Bejing, China* (Abstr. VIII-P #21, p280).

Flipsen, J.T.M., Van Lent, J.W.M., Goldbach, R.W., and Vlak, J.M. (1993). Expression of polyhedrin and p10 in the midgut of AcMNPV infected *Spodoptera exigua* larvae: an immunoelectron microscopic investigation. *Journal of Invertebrate Pathology* 61: 17-23.

Francki, R.I.B., Fauquet, C.M., Knudson, D. and Brown, F. (eds) (1991). Classification and nomenclature of viruses. Fifth Report of the International Committee on Taxonomy of Viruses. *Archives of Virology*, supplementum 2.

Gelernter, W.D and Federici, B.A. (1986a). Isolation, identification and determination of virulence of a nuclear polyhedrosis virus from the beet army worm, *Spodoptera exigua* (*Lepidoptera*: *Noctuidae*). *Environmental Entomology* 15: 240-245.

Gelernter, W.D. and Federici, B.A. (1986b). Continuous cell line from *Spodoptera exigua* (*Lepidoptera*: *Noctuidae*) that supports replication of nuclear polyhedrosis virus from *Spodoptera exigua* and *Autographa californica*. *Journal of Invertebrate Pathology* 48:199-207.

Goedert, M, Crowther, R.A. and Garner, C.C. (1991). Molecular characterization of microtubule-associated proteins tau and MAP2. *Trends in Neuroscience.* 14: 193-199.

Groebe, D.R., Chung, A.E. and Ho, C. (1990). Cationic lipid-mediated co-transfection of insect cells. *Nucleic Acids Research* 18: 4033.

Hink, F. (1970). Established insect cell line from the cabbage looper, *Trichoplusia ni*. *Nature* (London) 226: 446-467.

Kuzio, J., Rohel, D.Z., Curry, C.J., Krebs, A., Carstens, E.H. and Faulkner, P. (1984). Nucleotide sequence of the p10 polypeptide gene of *Autographa californica* nuclear polyhedrosis virus. *Virology* 139: 414-418.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) 227: 680-685.

Leisy, D.J., Rohrmann, G.F., Nesson, M. and Beaudreau, G.S. (1986). Nucleotide sequencing and transcriptional mapping of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus p10 gene. *Virology* 163: 157-167.

Quant-Russell, R.L., Pearson, M.N., Rohrmann, G.F. and Beaudreau, G.S. (1987). Characterization of baculovirus p10 synthesis using monoclonal antibodies. *Virology* 160: 9-19.

Quant-Russell, R.L., Pearson, M.N. and Rohrmann, G.F. (1991). Immunoelectron microsopic examination of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus-infected *Lymantria dispar* cells: time course and localization of major polyhedron-associated proteins. *Journal of General Virology* 72: 275-283.

Rohrmann, G.F. (1986). Polyhedrin structure. *Journal of General Virology* 73: 749-761.

Smith, G.E. and Summers, M.D. (1978). Analysis of baculovirus genomes with restriction enzyme endonucleases. *Virology* 98: 517-527.

Smith, G.E., Vlak, J.M. and Summers, M.D. (1983). Physical analysis of *Autographa californica* nuclear polyhedrosis virus transcripts for polyhedrin and 10,000 -molecular-weight protein. *Journal of Virology* 45: 215-225.

Smits, P.H. and Vlak, J.M. (1988). Quantative and qualitative aspects in the production of a nuclear polyhedrosis virus in *Spodoptera exigua* larvae. *Annales of Applied Biology* 112: 249-257.

Van der Wilk, F., Van Lent, J.W.M. and Vlak, J.M. (1987). Immunogold detection of polyhedrin, p10 and virion antigens in *Autographa californica* nuclear polyhedrosis virus-infected *S. frugiperda* cells. *Journal of General Virology* 68: 2615-2623.

Van Lent, J.W.M., Groenen, J.T.M., Klinge-Roode, E.C., Rohrmann, G.F., Zuidema, D., and 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., Sliwinsky, E.L., Goldbach, R.W. and Vlak, J.M. (1993). Functional domains of the p10 protein of *Autographa californica* nuclear polyhedorsis virus. *Journal of General Virology* 74: 563-574.

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

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klinge-Roode, E.C., Geervliet, J.B.F., Roosien, J. and Van Lent, J.W.M. (1988). Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-ß-galactosidase fusion gene. *Journal of General Virology* 69: 765-776.

Vlak, J.M., Schouten, A., Usmany, M., Belsham, G.J., Klinge-Roode, E.C., Maule, A.J., Van Lent, J.W.M. and Zuidema, D. (1990). Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. *Virology* 179: 312-320.

Volkman, L.E. and ZaaL, K.J.M. (1990). *Autographa californica* M nuclear polyhedrosis virus: microtubules and replication. *Virology* 175: 292-302.

Williams, G.V., Rohel, D.Z., Kuzio, J. and Faulkner, P. (1989). A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *Journal of General Virology* 70: 187-202.

Wilson, J.A., Hill, J.E., Kuzio, J and Faulkner, P. (1992). Nucleotide sequence of *Choristoneura fumiferana* nuclear polyhedrosis virus p10 gene (unpublished data). GenBank, Accession number M98513.

Yaozhou, Z. (1992). Studies on the p10 gene of *Bombyx mori* nuclear polyhedrosis virus. Chinese Academy of Agricultural Sciences PhD. Thesis. Graduate School of Chinese Academy of Agricultural Sciences, Shanghai Institute of Biochemistry Academia Sinica.

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

CARBOXY-TERMINAL SERINES OF *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS P10 PROTEIN ARE NOT INVOLVED IN FIBRILLAR STRUCTURE FORMATION

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SUMMARY

The p10 protein of *Autographa californica* nuclear polyhedrosis virus contains two serine residues in the carboxy-terminal region that can function as substrate for cAMP-dependent protein kinase (Cheley *et al.*, 1992). Previously, it was shown that the carboxy-terminal eight amino acids of p10 (RRGKRSSK) are involved in the formation of fibrillar structures in the nucleus and cytoplasm of infected insect cells (Van Oers *et al.*, 1993). To examine the role of the serine residues in this domain in the assembly of fibrillar structures, mutant baculoviruses were constructed that encoded p10 proteins in which either one or both of these serines were replaced by alanine residues. In cells infected with these mutants fibrillar structures were normally formed, both in the nucleus and the cytoplasm, and these structures had the same morphology as in wild type virus-infected cells. It is concluded, that these serines are not necessary to obtain fibrillar structures. Furthermore, the release of polyhedra from infected cells was not hampered in these mutants, confirming previous results that the fifteen carboxy-terminal amino acids of p10 are not involved in this process. Fibrillar structures of mutant p10 remained associated with electron-dense spacers and with polyhedral envelopes. This is also in agreement with previous results, which suggested that p10 has a binding site for the polyhedrin envelope protein and that this site is not located in the fifteen carboxy-terminal amino acids.

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INTRODUCTION

Nuclear polyhedrosis viruses (NPVs), belonging to the family *Baculoviridae* (Francki *et al.*, 1991), encode two proteins that are expressed at high level late in infection: polyhedrin (33 kDa), which is the major component of viral occlusion bodies and a protein of approximately 10 kDa, denoted p10. P10 proteins of several NPV species are found associated with fibrillar structures, that are formed both in the nucleus and cytoplasm of infected insect cells (Van der Wilk *et al.*, 1987; Quant-Russell *et al.*, 1987). Deletion of p10 indicated that p10 is not essential for virus replication neither *in vivo* nor *in vitro* (Vlak *et al.*, 1988), and this protein is involved in the release of polyhedra from infected cells (Williams *et al.*, 1989) by disintegration of the cell nuclei (Van Oers *et al.*, 1993). The presence of an intact p10 protein may result in a more effective release of individual polyhedra from insect cadavers and hence enhance the spread of virus into the environment. The intimate structural relation between polyhedral envelopes and the polyhedron matrix seems to be reduced in p10 deletion mutants, which may affect the stability of the polyhedra (Williams *et al.*, 1989; Gross *et al.*, 1994). P10 protein sequences have apparently diverged considerably during NPV evolution, but the gene as such, the size of the protein and its secondary structure are conserved (Zuidema *et al.*, 1993; Van Oers *et al.*, 1994). All known p10 proteins have in common a carboxy-terminal domain with several positively charged amino acids and at least one serine residue (Van Oers *et al.*, 1994).

Studies with p10 deletion mutants have revealed that fibrillar structure formation and disintegration of infected-cell nuclei are two functions located in separate domains of p10. Deletion of the eight carboxy-terminal amino acids (RRGKRSSK) from the *Autographa californica* (Ac) MNPV p10 protein, revealed that this sequence is vital for fibrillar structure formation but not for nuclear disintegration (Van Oers *et al.*, 1993). The nuclear disintegration domain is likely to located between amino acids 52 and 75, and an aggregation function was postulated for the amino-terminal half of this protein (Vlak *et al.* 1988; Van Oers *et al.*, 1993). When the carboxy-terminal domain was removed, p10 molecules still aggregated in nucleus and cytoplasm, but did not assemble into fibrillar structures. The mechanism of the alignment of p10 aggregates into fibrillar structures is not known.

Peptides with the consensus sequence RRXSX or RXXSX, where X can be any amino acid, are known as substrates for cAMP-dependent protein kinase (PKA) (Edelman *et al.*, 1987; Steiner *et al.*, 1990). The carboxy-terminus of p10 ($KRS_{92}S_{93}KK$) resembles this consensus. A recombinant baculovirus expressing the catalytic subunit of a PKA from *Aplysia californica* induced long cell processes in infected insect cells (Cheley and Bayley, 1991; Cheley *et al.*, 1992). In these cells p10 was phosphorylated on one of the carboxyl-terminal serine residues. This type of phosphorylation increased the affinity of p10 for microtubules.

Some co-localization of p10 with microtubules was present in the cytoplasm of wild type (wt) AcMNPV infected cells (Volkman and Zaal, 1991).

The increased affinity of p10 for microtubules upon phosphorylation prompted us to investigate the possible role of the carboxy-terminal serines in the formation of fibrillar structures in a wild type (wt) AcMNPV infection. Therefore, mutant p10 sequences encoding alanine instead of serine at postions 92 or 93, or at both positions, were constructed by PCR methodology. This eliminates the exposure of the hydroxyl group of serine, which is sensitive to phosphorylation by PKA. The consequences of these mutations for the replication and cytopathology were analysed.

MATERIALS AND METHODS

Cells, virus isolates and recombinant AcMNPV viruses

The *Spodoptera frugiperda* cell line IPLB-SF-21 (Vaughn *et al.*, 1977) was used and maintained in TNM-FH medium (Hink, 1970) supplemented with 10% fetal bovine serum. The AcMNPV strain E2 was used as wt virus (Smith and Summers, 1978). The AcMNPV p10 deletion mutant AcMO21 was used as parental virus in recombination experiments. In this mutant the p10 coding sequence has been replaced with a linker sequence containing a *Bsu*36I restriction site (Martens *et al.*, 1994). This unique *Bsu*36I site in the genome of AcMO21 enables linearization of the viral DNA and facilitates the retrieval of recombinants. Two AcMNPV recombinants were used as controls in the mutant analysis: AcMO16, containing a wt p10 coding sequence flanked by *Bam*HI sites, and AcMO19, encoding a p10 protein which lacks the eight carboxy-terminal amino acids (Fig. 6.1; Van Oers *et al.*, 1993). The molecular context of the p10 coding sequence in these controls was the same as in the mutants described below.

Construction of transfer vectors with mutant p10 coding sequences

Mutated p10 sequences, encoding p10 proteins in which either Ser92 or Ser93 or both Ser92 and Ser93 were changed into Ala, were constructed with PCR technology. The AcMNPV *Eco*RI-P clone pAc159R (Smith *et al.*, 1983) was used as source for the wild type p10 gene. The p10 sequence was synthesized from positions -3 to +282, encompassing the entire coding sequence, three upstream nucleotides and the translational stop codon. The mutations were introduced by mismatches in the downstream primers (Table 6.1; Fig 6.1). To facilitate subsequent cloning *Bam*HI sites were introduced at both ends of the PCR products and therefor, all primers contained additional sequences at their 5' ends.

PCR reactions were performed in a volume of 20 μl containing 150 ng template DNA (pAc159R) and 12 pmol of both up- and downstream primer, 10 mM KCl, 10 mM

$(NH_4)_2SO_4$, 5 mM $MgSO_4$, 20 mM Tris-HCl, pH 8.8; 0.2% Triton X-100, 4 μM of each deoxynucleotide and 2 units Vent(exo-) DNA polymerase (New England Biolabs). An overlay of 10 μl mineral oil was applied to prevent evaporation. Thirty cycles were performed (Perkin-Elmer Step cycle device) of 1 min denaturation at 95 °C, 1 min annealing at 30°, heating up to 72° in 1 min 30 sec, and further elongation at 72°C for 2 min. After chloroform extraction to remove residual mineral oil, the product was precipitated and digested with *Bam*HI (Gibco BRL). The digested PCR product was separated from the primers and the template by electrophoresis in a 1% agarose gel, recovered by the freeze-squeeze method (Tautz *et al.*, 1983) and cloned into pUC19 to enable easy screening for plasmids containing the PCR product. This procedure resulted in the plasmids pAcBA1 (Ala92), pAcBA2 (Ala93) and pAcBA3 (Ala92,93). Subsequently, the *Bam*HI fragments containing the p10 sequences were inserted into the p10 promoter-based transfer vector pAcAS3 (Vlak *et al.*, 1990), resulting in the transfer vectors pAcBA4 (Ala92), pAcBA5 (Ala93) and pAcBA6 (Ala92,93). The authenticity of the modified p10 coding sequences were ascertained by DNA sequencing with a p10 promoter-specific primer (5' AATTATT-ATCAAATCATTTG 3') homologous with the coding strand from position -68 to -48.

Table 1. Primers used for PCR*

Description	Nucleotide sequence 5' → 3'
Upstream primer	**GGGGATCC**atcatgtcaaagccta
Downstream primer 1 (Ala92)	**GGGGATCC**ttacttggaa**GC**gcgtttaccacgacg
Downstream primer 2 (Ala93)	**GGGGATCC**ttacttgg**C**actgcgtttaccacgacg
Downstream primer 3 (Ala92,93)	**GGGGATCC**ttacttgg**CaGC**gcgtttaccacgacg
Downstream primer 4 (wild type p10)	**GGGGATCC**ttacttggaactgcgtttaccacgacg

*Nucleotides printed in upper case represent nucleotides not homologous to the template DNA (pAcR159). The primer 4 has been used to make a full length p10 coding sequence flanked by *Bam*HI sites (van Oers *et al.*, 1993) and was included for comparison.

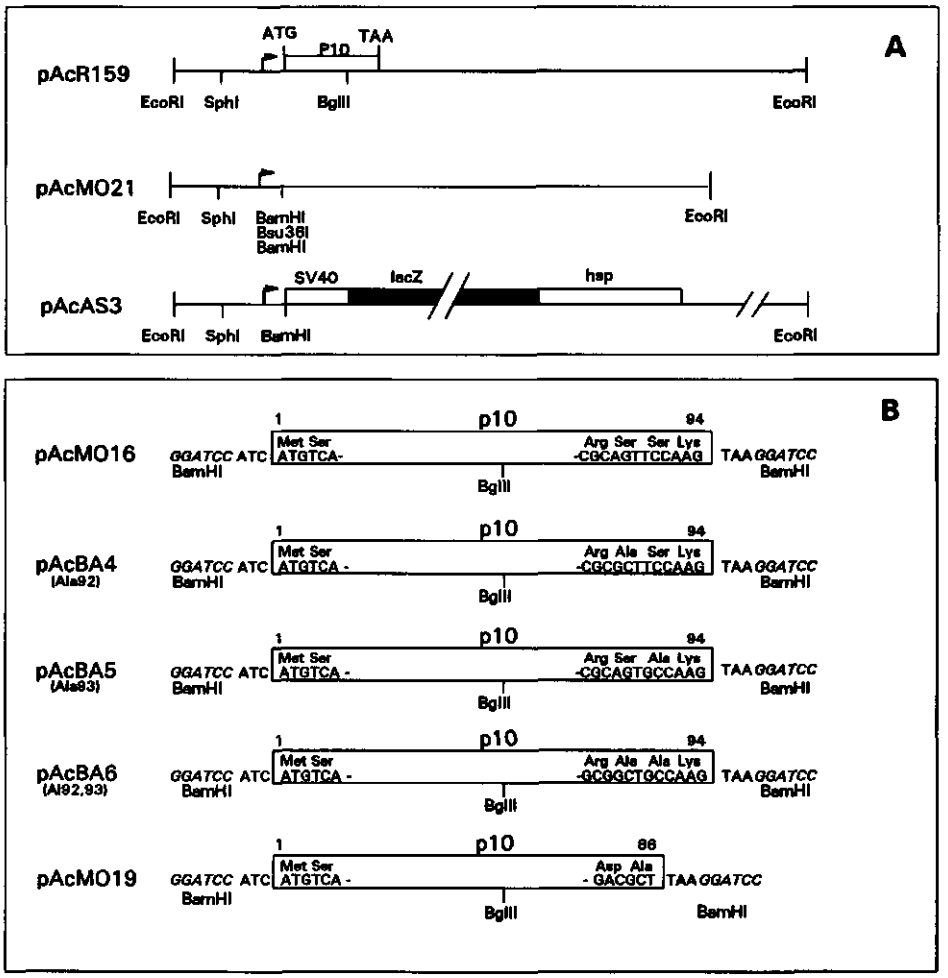

Figure 6.1: A) Schematic representation of the *Eco*RI-P fragment of AcMNPV in plasmids pAc159R (Smith *et al.*, 1983), containing the p10 gene and the same fragment in pAcMO21 (Martens *et al.*, 1994) and pAcAS3 (Vlak *et al.*, 1988). pAc159R was used as source for the wt p10 gene. The transfer vector pAcMO21 was used to construct a p10 deletion mutant with a unique *Bsu*36I site (Martens *et al.*, 1994). This virus was used as parental virus in recombination experiments. Plasmid pAcAS3 was used as parental transfer vector for insertion of mutated p10 sequences. B) Representation of the wt p10 coding sequence as present in pAcMO16 and the mutated p10 coding sequences in the various transfer vectors. These vectors resulted from insertions of the *Bam*HI-fragments from PCR experiments (Materials and Methods) into the *Bam*HI site of pAcAS3. The modifications in the carboxy-terminal domain are indicated. Arrows indicate the transcriptional start site. Only restriction sites important for this study are indicated.

Transfection of DNA to SF-21 cells and selection of p10 mutant viruses

AcMO21 viral DNA was isolated from budded virions using standard methods (Summers and Smith, 1987) and linearized at the p10 locus with *Bsu*36I (New England Biolabs). Recombinant viruses were obtained by lipofectin-mediated co-transfection (Groebe *et al.*, 1990) of 1 μg transfer vector (pAcBA4, pAcBA5 or pAcBA6) and 0.5 μg linearized AcMO21 viral DNA. Subsequently, the recombinants were selected and plaque purified by virtue of their 'blue' phenotype upon addition of X-gal, conferred by the hsp-lacZ-SV40 gene cassette present in the transfer vectors (Fig. 6.1).

DNA and protein analysis of p10 mutant viruses

SF-21 cells were infected with AcMO21, AcBA4, AcBA5 and AcBA6 and viral DNA was extracted from extracellular virus (ECV) at 72 h post infection (p.i.). The *Bgl*II restriction pattern was analysed in 0.7% agarose gels. The identity of the recombinant viruses was further verified by direct sequencing on DNA extracted from ECVs. Virus specific protein synthesis was analysed by infecting SF-21 cells at a multiplicity of infection (m.o.i.) of 20 $TCID_{50}$ units/cell with wt AcMNPV, AcMO21, AcBA4, AcBA5, AcBA6 or AcMO16. Infected cells were harvested at 46 h p.i. Protein samples were analysed on a 13.5% SDS-polyacrylamide gel (Laemmli, 1970) using a Bio-Rad Mini-Protean^{II} apparatus. Gels were either stained with Coomassie brilliant blue or subjected to immunoblot analysis as described previously (Van Oers *et al.*, 1993). Antiserum prepared in rabbits against AcMNPV p10 was a gift from dr P. Faulkner (Queen's University, Kingston, Ontario) and was used at a 1:1000 dilution in immunoblot analysis. Goat-anti-rabbit immunoglobulins conjugated to alkaline phosphatase (Tago) were used atindicate a 1:1000 dilution.

Computer analysis

Hydrophilicity values and surface predictions were calculated for the modified p10 proteins and compared to wt p10. The AcMNPV p10 sequence data were derived from Kuzio *et al.*, (1984) and adjusted for the introduced mutations. The methionine at position 1 was omitted The hydrophilicity values were calculated, according to the methods of Kyte and Doolittle (1982) and averaged over a window of seven amino acids. Surface probability predictions were calculated with the method of Emini *et al.* (1985), averaged over six amino acids. All calculations were performed with programs developed by the University of Wisconsin Genetics Computer Group (UWGCG version 7). Hydrophilicity graphs and surface probability profiles were plotted for the carboxy-terminal 25 amino acids using LOTUS and FREELANCE programs (Lotus Development Company).

Electron microscopy and immunogold labelling

SF-21 cells were infected with the p10 mutants at a m.o.i of 10 $TCID_{50}$ units/cell. Cells were harvested 46 h p.i. and fixed and embedded in LR Gold resin (London Resins Company) as described by Van Lent *et al.* (1990) and modified according to Van Oers *et al.* (1993). Ultrathin sections were cut with a diamond knife and mounted on nickel grids coated with a formvar supporting film. Immunogold labelling was performed as described by Van Lent *et al.* (1990) with 1:1000 diluted anti-p10 serum (see above) and subsequently with protein A-gold with a diameter of 7 nm. Immunogold-labelled sections were stained as described by Flipsen *et al.* (1992) and examined in a Philips CM12 transmission electron microscope.

Polyhedron release studies

To study the release of polyhedra from infected cells, Sf-21 cells were infected at a m.o.i. of 10 $TCID_{50}$ units/cell with AcMNPV wt, AcMO21 and the various mutants. Infected cell cultures were examined 5 days after infection by phase contrast microscopy.

RESULTS

Construction of AcMNPV mutants encoding mutated p10 proteins

A deletion of the eight carboxy-terminal amino acids (RRGKRSSK) from AcMNPV p10 resulted in the disappearance of fibrillar structures and aggregates of p10 were observed in the nucleus and the cytoplasm (Van Oers *et al.*, 1993). The positively-charged carboxy terminus of p10 is conserved in NPVs as is the presence of at least one serine residue in this part of p10. To analyse the function of these serines in fibrillar structure formation, either one or both serine residues were replaced with alanine thereby eliminating the hydroxyl groups prone to phosphorylation (Cheley *et al.*, 1992). The effect of these amino acid replacements on the hydrophilicity profile and surface probability of the carboxy terminus of p10 was calculated (Fig. 6.2) with the p10 protein in an unphosphorylated state. The results indicate that the hydrophilicity is less prominent than in wt p10. However, the probability that the carboxy-terminal domain is exposed at the surface of the protein is not drastically altered by modifying the serine residues.

Three p10 mutant AcMNPV viruses were obtained by recombination using transfer vectors that carried the mutated p10 sequences (see Materials and Methods; Fig. 6.1) : mutant AcBA4 (Ser92→Ala92), AcBA5 (Ser93→Ala93) and AcBA6 (Ser92,93→Ala92,93). The identity of the mutant viruses was confirmed by restriction enzyme analysis and DNA sequencing (data not shown). Mutant AcMO16 (Fig. 6.1; Van Oers *et al.*, 1993) had a wt p10 coding sequence in the same sequence context as the three p10 mutants and was used as a positive control. Recombinant AcMO19 (Fig. 6.1; Van Oers *et al.*, 1993) expressed a p10

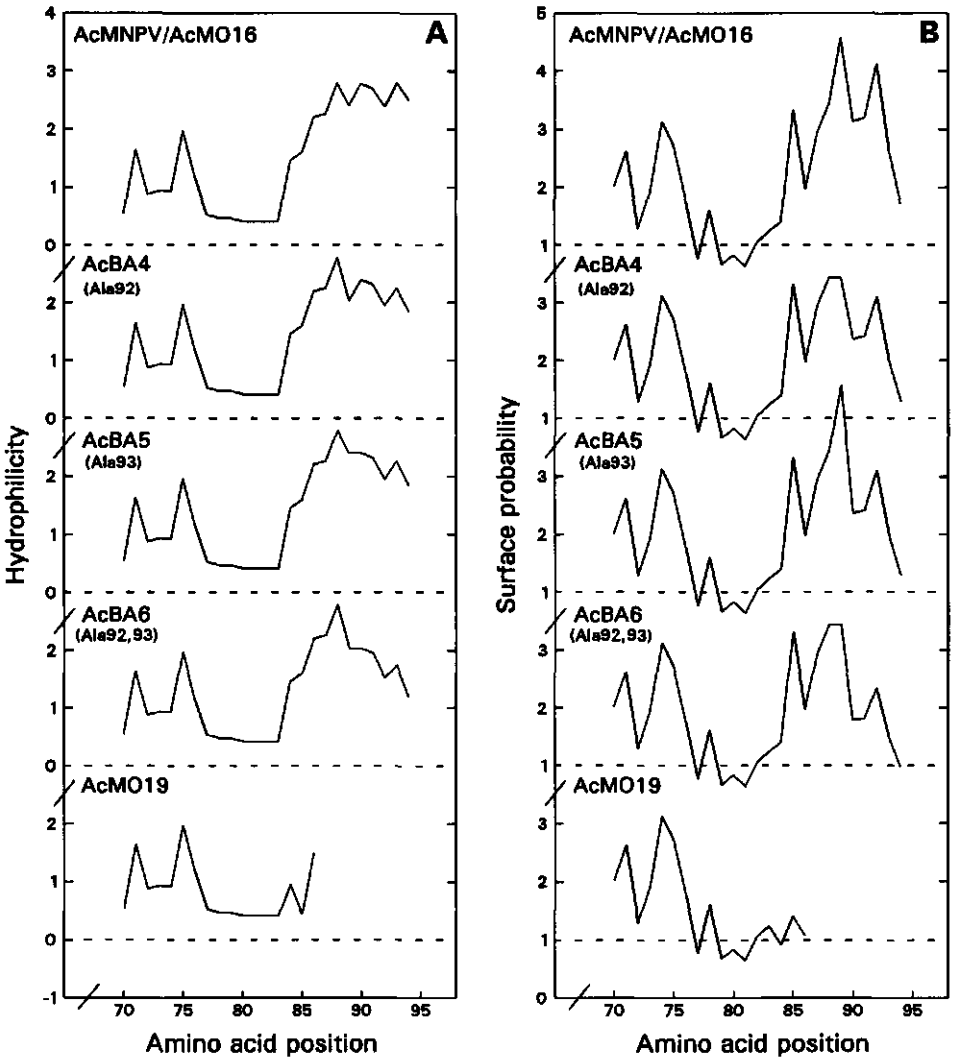

Figure 6.2: A) Hydrophilicity profiles for wt AcMNPV p10 and the various mutant p10 proteins. These profiles were calculated with the method of Kyte and Doolittle (1982) averaged over a window of seven amino acids. Possitive values indicate hydrophilicity, negative values mean hydrophobicity. B) Surface probability predictions for wt p10 and the modified p10 proteins, calculated with the method of Emini *et al.* (1985) over a window of six amino acids. Values above 1.0 indicate a more than average probability to be at the surface of a protein. The p10 sequence was derived from Kuzio *et al.* (1984). Since the differences occur in the carboxy-terminal part, only the values for the carboxy-terminal 25 amino-acids are indicated.

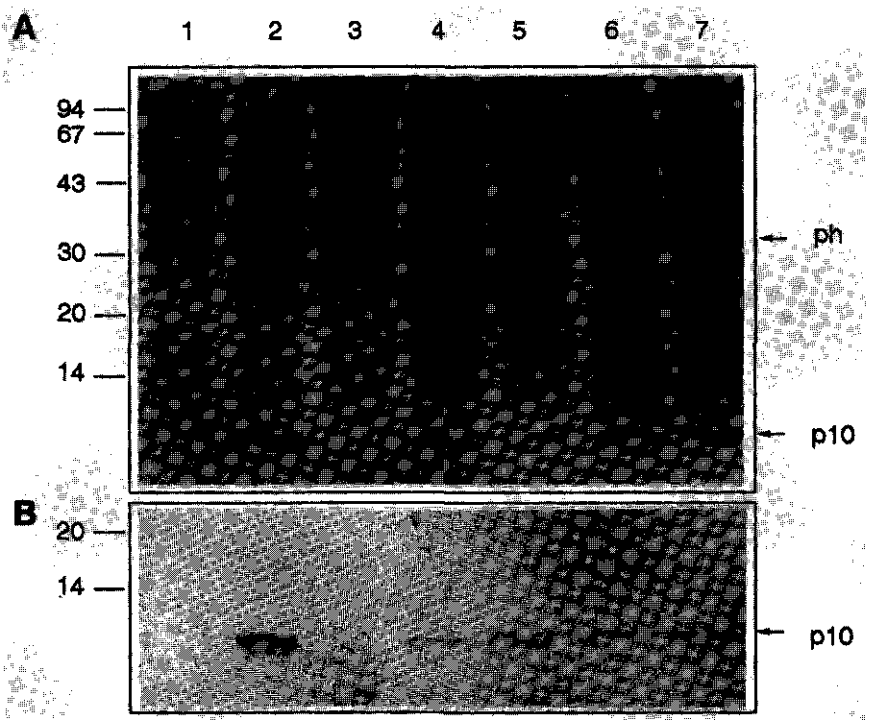

Figure 6.3: A) SF-21 cells (1) infected with wt AcMNPV (2), the p10 deletion mutant AcMO21 (3), the p10 mutants AcBA4 (4), AcBA5 (5) and AcBA6 (6), and the control mutant AcMO16 (7) were harvested 46 h p.i and analysed in 13.5% SDS-PAGE. Each lane contains the equivalent of 6.7 x 10^4 SF-21 cells. Molecular weigth markers are indicated in kDa. B) Immuno blot analysis of (A) using polyclonal p10 antiserum.

protein lacking the eight carboxy-terminal amino acids and was used as negative control for fibrillar structure formation.

P10 expression in cells infected with mutant viruses

The expression of mutant p10 proteins in insect cells was analysed for the various mutant viruses by SDS-PAGE at 46 h p.i (Fig. 6.3). In contrast to wt AcMNPV (Fig 6.3 A; lane 2), cells infected with the parental virus AcMO21 (lane 3) did not synthesize p10 protein, as expected. Cells infected with the mutant viruses AcBA4 (lane 4), AcBA5 (lane 5) and AcBA6 (lane 6) or the control mutant AcMO16 (lane 7) made a protein of the same size as with wt AcMNPV (10 kDa). The identity of these proteins as p10-derived polypeptides was confirmed by immunoblot analysis using AcMNPV p10-specific polyclonal antiserum

(Fig. 6.3 B). These results indicated that the mutations did not affect the electrophoretic mobility of p10 protein.

Cytopathology of p10 mutants

P10 is involved in the disintegration of baculovirus-infected cell nuclei, resulting in the release of polyhedra (Van Oers *et al.*, 1993). To determine whether the liberation of polyhedra from infected cell nuclei was affected by the mutations in p10, cells were infected and examined five days after infection by light microscopy. In infections with all three mutants high levels of free polyhedra were present in the culture medium, showing that these mutants were perfectly capable of releasing polyhedra and that the carboxy-terminal serines are not involved in this process. This confirmed previous experiments where this capacity was retained even when up to fifteen amino acids were removed from the carboxy terminus of p10 (Van Oers *et al.*, 1993).

To analyse whether the modified p10 proteins associated into fibrillar structures, infected cells were collected 46 h p.i. and prepared for electron microscopy (Fig. 6.4). In cells infected with mutant AcBA4 (Ala92; Fig. 6.4 A), AcBA5 (Ala93; Fig. 6.4 B) and AcBA6 (Ala92,93; Fig. 6.4 C) fibrillar structures were present in the nucleus and cytoplasm. These fibrillar structures had the same morphology as in AcMO16 (Fig. 6.4 D) or wt AcMNPV-infected cells. This is in contrast with AcMO19-infected cells, where truncated p10 polypeptides existed in an aggregated form, but did not form fibrillar structures (van Oers *et al.*, 1993). That p10 polypeptides assembled efficiently into fibrillar structures was confirmed by immunogold labelling, since no p10 was detected outside fibrillar structures. The results indicate that the hydroxyl groups on Ser92 and 93 can be omitted for fibrillar structure formation both in the nucleus and the cytoplasm, and therefore, that phosphorylation of p10 is not required in this process.

In wt AcMNPV polyhedra are surrounded by an electron-dense layer called the polyhedron envelope or polyhedron calyx. A phosphoprotein called the polyhedron envelope protein (pp34) is essential for its formation (Zuidema *et al.* , 1989). For proper polyhedron envelope formation p10 also seems to be required (Williams *et al.*, 1990; Gross *et al.*, 1994). The mutants described here made normal polyhedra that were surrounded by a polyhedron envelope as seen on ultrathin sections (Fig. 6.4), as was the mutant lacking eight carboxy-terminal amino acids (AcMO19). From these data however we could not conclude whether "holes", as described by Gross *et al.*, (1994), were present on the surface of the polyhedron envelope.

Electron-dense spacers also contain polyhedron envelope protein and these structures are often formed at the edge of fibrillar structures, suggesting that p10 has a binding site for this protein. In cells infected with the mutants AcBA4, AcBA5 and AcBA6 polyhedron envelopes

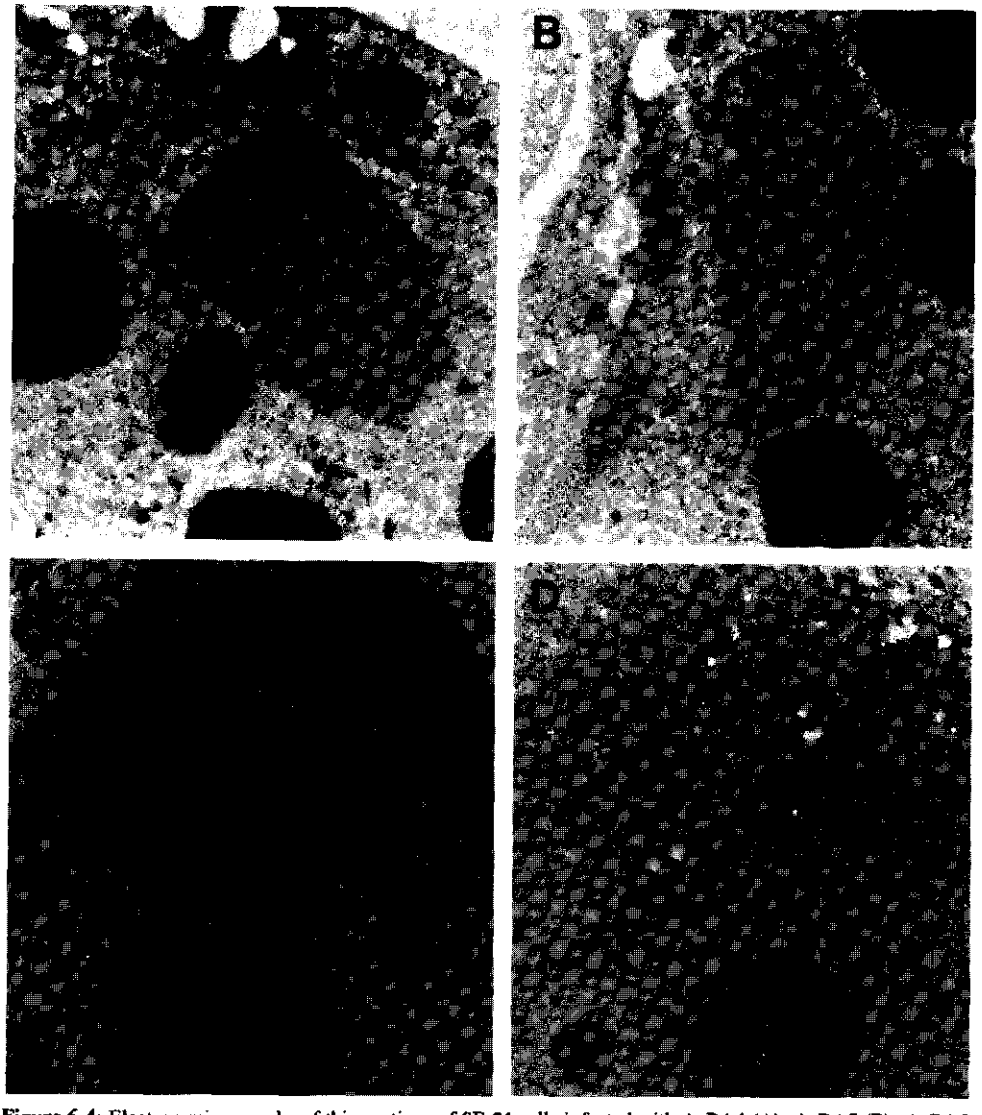

Figure 6.4: Electron micrographs of thin sections of SF-21 cells infected with AcBA4 (A), AcBA5 (B), AcBA6 (C) and AcMO19 (D) were harvested at 46 h p.i. The sections were treated with p10 antiserum and subsequently complexed to protein A-gold to detect p10 protein. Fibrillar structures (F) in nucleus and cytoplasm are indicated. The bar marker represents 0,5 μm.

and electron-dense spacers were normally formed often in close association with fibrillar structures (Fig. 6.4). This contrasted with AcMO19-infected cells that did not form electron-dense spacers. The absence of spacers in AcMO19-infected cells was not simply due to an intact p10, since AcAS3, a p10 deletion mutant, was able to induce these structures (Van Oers *et al.*, 1993).

DISCUSSION

The positively-charged nature of the carboxy terminus of p10 is conserved in all NPV species analysed thus far, as is the presence of at least one serine-hydroxyl group in this part of p10 (Van Oers *et al.*, 1994). Deletion mutagenesis of the carboxy terminus of AcMNPV p10 showed that the amino acid sequence RRGKRSSK is involved in the formation of fibrillar structures (Van Oers *et al.*, 1993). Without this domain p10 molecules aggregate in the nucleus and cytoplasm, but do not assemble into fibrillar structures. The exact mechanism, that converts p10 aggregates into fibrillar structures is still enigmatic, but their are some indications that tubulin is involved in this process. In the case of *Orgyia pseudotsugata* (Op) MNPV infecting *Lymantria dispar* cells, it has been suggested that p10 could be responsible for reorganizing microtubules (MTs) in NPV-infected cells, since cross reactivity of a monoclonal antibody against OpMNPV p10 was observed with a microtubular component in uninfected cells (Quant-Russell *et al.*, 1987). Further evidence to support this hypothesis has not been obtained, but there are indications that p10 interacts with residual MTs. Immunofluorescence studies of wt AcMNPV-infected cells revealed that MTs co-localizing with p10 formed much thicker threads than those that did not, suggesting a MT cross-linking or bundling function for p10 (Volkman and Zaal, 1991).

Recently, the catalytic subunit of cAMP-dependent protein kinase (PKA) derived from the mollusk *Aplysia californica* was expressed in a recombinant baculovirus (Cheley and Bayley, 1991). Cells infected by this recombinant formed long cell processes with a typical morhology. The p10 protein was phosphorylated on the carboxy-terminal pentapeptide (KRSSK) (Cheley *et al.*, 1992) and this resulted in an enhanced affinity of p10 for MTs. The p10 protein behaved as a heat-stable microtubule-associated protein. Although phosphorylated p10 had an enhanced affinity for MTs, it is not known whether phosphorylated p10 is the agent that induces the cell processes. Experiments have been initiated to construct recombinant viruses, that express PKA and have a deletion in the p10 sequence or encode a p10 protein with alanines in place of the carboxy-terminal serine residues.

Although phosphorylation of p10 in wt AcMNPV-infected cells could not be detected (Cheley *et al.*,1992), it might be that phosphorylation of p10 at a low level results in cross-linking aggregates of p10 molecules to MTs or to tubulin dimers. In that case removal of the

hydroxyl groups from the carboxy-terminal serines, thereby preventing phosphorylation, would result in aggregated p10 proteins, as when the carboxy-terminus is removed (AcMO19). Instead we observed that fibrillar structure formation occurred normally in the absence of those hydroxyl groups (Fig. 6.4). These results indicate that phosphorylation of p10 does not play an essential role in fibrillar structure formation.

In SF-9 or SF-21 cells endogenous PKA, when present, is likely to exist in an inactive form, keeping p10 in an unphosphorylated state. This contrasts with the situation in cells infected with recombinant virus, expressing the catalytic-subunit of PKA at high levels as described by Cheley and Bayley (1991). In insects, however, endogenous PKAs might become activated by hormones, which affect the cAMP levels in the cells. In that case phosphorylated p10 may alter the organization of MTs in the insect. PKAs are highly conserved proteins within the animal kingdom (for review see Edelman *et al*, 1987) and the gene for the catalytic subunit of PKA from *Drosophila* has been reported (Foster *et al.*, 1988; Kalderon and Rubin, 1988).

Both α- and ß- tubulin molecules contain acidic carboxy-terminal domains, which are most likely involved in binding the microtubule-associated proteins at basic amino acid repeats in their carboxy-terminal halfs (Lee, *et al.*, 1988; Lewis *et al.*, 1988; Goedert *et al.*, 1991). Only limited sequence homology exists between AcMNPV p10 and those basic repeats plus the sequence just upstream (Cheley *et al.*, 1992). However, the acidic domains in tubulin might still be the targets for the basic carboxy termini of p10 molecules. Mutations in the Arg and Lys residues in the carboxy terminus of p10 could alter this interaction. Adressing the question whether such an interaction takes place in wild type-infected cells needs biochemical analysis of fibrillar structures. Since tubulin is not present at a detectable level in the nucleus of eukaryotic cells, it remains questionable whether nuclear p10 can interact with tubulin.

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REFERENCES

Cheley, S. and Bayley, H. (1991). Kinetics and regulation of two catalytic subunits of cAMP-dependent protein kinase from *Aplysia californica*. *Biochemistry* 30: 10246-10255.

Cheley, S., Kosik, K.S., Paskevich, P., Bakalis, S. and Bayley, H. (1992). Phosphorylated baculovirus p10 is a heat stable microtubule-associated protein associated with process formation in SF-9 cells. *Journal of Cell Science* 102: 739-752.

Edelman, A.M., Blumenthal, D.K., and Krebs, E.G. (1987). Protein serine/threonine kinases. *Annual Review of Biochemistry* 56: 567-613.

Emini, E.A., Hughes, J.V., Perlow, D.S. and Boger, J. (1985). Induction of hepatitis A virus-neutralizing antibody by a virus specific synthetic peptide. *Journal of Virology* 55: 836-839.

Flipsen, J.T.M., Van Lent, J.W.M., Goldbach, R.W. and Vlak, J.M. (1992). Expression of polyhedrin and p10 in the midgut of AcMNPV infected *Spodoptera exigua* larvae: an immunoelectron microscopic investigation. *Journal of Invertebrate Pathology* 61: 17-23.

Foster, J.L., Higgins, G.C. and Jackson, F.R (1988). Cloning, sequence and expression of the *Drosophila* cAMP-dependent protein kinase catalytic subunit. *Journal of Biological Chemistry* 263: 1676-1681.

Francki, R.I.B., Fauquet, C.M., Knudson, D. and Brown, F. (eds) (1991). Classification and nomenclature of viruses. Fifth Report of the International Committee on Taxonomy of Viruses. *Archives of Virology* supplementum II: pp 450.

Goedert, M., Growther, R.A. and Garner, C.C. (1991). Molecular characterization of microtubule-associated proteins tau and MAP2. *Trends in Neuroscience* 14: 193-199.

Groebe, , D.R., Chung, A.E. and Ho, C. (1990). Cationic lipid-mediated co-transfection of insect cells. *Nucleic Acids Rresearch* 18: 4033.

Gross, C.H., Russell, R.L.Q. and Rohrmann, G.F. (1994). The *Orgyia pseudotsugata* baculovirus p10 and polyhedron envelope protein gene: analysis of their relative expression levels and role in polyhedron structure. *Journal of General Virology* 75: 000-000.

Hink, F. (1970). Established insect cell line from the cabbage looper, *Trichoplusia ni. Nature (London)* 226: 446-467.

Kalderon, D. and Rubin, G.M. (1988). Isolation and characterization of *Drosophila* cAMP-dependent protein kinase genes. *Genes and Development* 2, 1539-1556.

Kitts, P.A., Ayres, M.D. and Possee, R.D. (1990). Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic Acids Research* 18: 5667-5672.

Kuzio, J., Rohel, D.Z., Curry, C.J., Krebs, A., Carstens, E.H. and Faulkner, P.(1984). Nucleotide sequence of the p10 polypeptide gene of *Autographa californica* nuclear polyhedrosis virus. *Virology* 139: 414-418.

Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydrophobic character of a protein. *Journal of Molecular Biology* 157: 105-132.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bateriophage T4. *Nature (London)* 227: 680-685.

Lee, G., Cowan, N. and Kirschner, M. (1988). The primary structure and heterogeneity of tau protein from mouse brain. *Science* 239: 285-288.

Lewis, S.A, Wang, D. and Cowan, N.J. (1988). Microtubule-associated protein MAP2 shares a microtubule binding motif with tau protein. *Science* 242: 936-939.

Martens, J.W.M., Van Oers, M.M., Van de Bilt, B., Vlak, J.M. and Oudshoorn, P. (1994). Efficient recovery of baculovirus recombinants expressing foreign genes under p10 promoter control *Bio/Techniques* (submitted).

Quant-Russell, R.L., Pearson, M.N., Rohrmann, G.F. and Beaudreau, G.S. (1987). Characterization of baculovirus p10 synthesis using monoclonal antibodies. *Virology* 160: 9-19.

Qaunt-Russel, R.L., Pearson, M.N. and Rohrmann, G.F. (1991). Immunoelectron examination of *Orgyia pseudotsugata* nuclear polyhedrosis virus-infected *Lymantria dispar* cells: time course and localization of major polyhedron-associated proteins. *Journal of General Virology* 72: 275-283.

Smith, G.E. and Summers, M.D. (1978). Analysis of baculovirus genomes with restriction enzyme endonucleases. *Virology* 98: 517-527.

Smith, G.E., Vlak, J.M. and Summers, M.D. (1983). Physical analysis of *Autographa californica* nuclear polyhedrosis virus transcripts for polyhedrin and 10,000-molecular-weight protein. *Journal of Virology* 45: 215-225.

Steiner, B., Mandelkow, E.M., Biernat, J., Gustke, N., Meyer, H.E., Schmidt, B., Mieskes, G., Sölling, H.D., Drechsel, D., Kirschner, M.W., Goedert, M. and Mandelkow, E. (1990). Phosphorylation of microtubule associated protein tau: identification of the site for Ca^{2+}-calmodulin dependent kinase and relationship with tau phosphorylation in Alzheimer tangles. *The EMBO Journal* 9: 3539-3544.

Summers, M.D. and Smith, G.E. (1987). A manual of methods for baculovirus vectors and insect cell cultures procedures. *Texas Agricultural Experiment Station Bulletin* No.1555.

Tautz, D. and Rentz, M. (1983). An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. *Analytical Biochemistry* 132: 14-19.

Van der Wilk, F., Van Lent, J.W.M. and Vlak, J.M. (1987). Immunogold detection of polyhedrin, p10 and virion antigens in *Autographa californica* nuclear polyhedrosis virus-infected *S. frugiperda* cells. *Journal of General Virology* 68: 2615-2623.

Van Lent, J.W.M., Groenen, J.T.M., Klinge-Roode, E.C., Rohrmann, G.F., Zuidema, D. and 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., Sliwinsky, E.L., Goldbach, R.W. and Vlak, J.M. (1993). Functional domains of the *Autographa californica* nuclear polyhedrosis virus p10 gene. *Journal of General Virology*, 74: 563-574.

Van Oers, M.M., Flipsen, J.T.M., Reusken, C.B.E.M. and Vlak, J.M. (1994). Specificity of baculovirus p10 functions. *Virology* 200: 000-000.

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

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klinge-Roode, E.C., Geervliet, J.B.F., Roosien, J. and Van Lent, J.W.M. (1988). Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-ß-galactosidase fusion gene. *Journal of General Virology* 69: 765-776.

Vlak, J.M., Schouten, A., Usmany, M., Belsham, G.J., Klinge-Roode, E.C., Maule, A.J., Van Lent, J.W.M. and Zuidema, D. (1990). Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene and a novel selection method. *Virology* 179: 312-320.

Volkman L.E. and Zaal, K.J.M. (1990). *Autographa californica* M nuclear polyhedrosis virus: microtubules and replication. *Virology* 175: 292-302.

Williams, G.V., Rohel, D.Z., Kuzio, J. and Faulkner, P. (1989). A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *Journal of General Virology* 70: 187-202.

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

Chapter 7

GENERAL DISCUSSION

INTRODUCTION

Nuclear polyhedrosis viruses (NPVs, *Baculoviridae*; Francki *et al.*, 1991) are characterized by the presence of rod-shaped virions containing covalently closed, double-stranded DNA, which are occluded in large nuclear-located protein crystals, called occlusion bodies or polyhedra. The matrix protein of these occlusion bodies, known as polyhedrin (approximately 30 kDa), is expressed at high levels in infected cells, as is the other very late expressed baculovirus protein, p10 (approximately 10 kDa). Both polyhedrin and p10 are dispensable for virus replication in cultured insect cells (Smith *et al.*, 1983; Vlak *et al.*, 1988). This enabled the exploitation of the polyhedrin and p10 locus as well as their promoters for the insertion and expression of foreign genes. Nowadays, NPVs have wide applications in areas such as insect control, veterinary and medical science, clinical diagnosis and biotechnology. At the same time, however, it is important to understand the basic functions of polyhedrin and p10, and the consequences of the absence of these proteins for the infection process and for persistence of NPVs in the environment. This is relevant when genetically modified viruses will be used in the field (Vlak, 1993). The function of polyhedra seems to protect virions in infected cells against proteolysis during morphogenesis and outside the host insect against environmental decay. Deletion of the polyhedrin gene results in viruses with highly reduced stability and persistence in the field. Polyhedra are therefore indispensable for use in insect control. P10 protein is associated with fibrillar structures present in the nucleus and cytoplasm of infected cells (Croizier *et al.*, 1987; Quant-Russell *et al.* 1987, Van der Wilk *et al.*, 1987). The function of these structures remains enigmatic and the consequences of p10 deletions for the persistence in the environment are not well understood.

The studies described in this thesis concern the exploitation of the p10 locus for foreign gene expression and the functional analysis of this highly expressed baculovirus protein. In this chapter the data described in the previous chapters are discussed and reflected against recent literature. A model is proposed presenting the current thinking on the functional domains of p10, and suggestions for further research regarding p10 are made.

Regulation of baculovirus gene expression occurs at the transcriptional level (Friesen and Miller, 1986). Promoters of baculovirus very late genes have a similar structure (Rohrmann, 1986; Zanotto *et al.*, 1993), and hence a similar pattern of gene regulation is expected for p10 and polyhedrin genes. The simultaneous activity of both the p10 and polyhedrin promoter might result in a competition for transcription factors, thereby reducing the expression levels of each individual gene. The results reported in Chapter 2 show that the activity of the *Autographa californica* (Ac) MNPV p10 promoter is not influenced by the presence or absence of a functional polyhedrin gene, suggesting some specificity in polyhedrin versus p10 gene expression. Time course experiments showed that the activity of both genes followed a different pattern over time (Roelvink *et al.*, 1992). A recent report by Chaabihi *et al.* (1993) confirmed our conclusion, that p10 expression is not increased upon deletion of the polyhedrin promoter. On the other hand, deletion of the p10 gene increased the transcriptional activity of the polyhedrin promoter four-fold (Chaabihi *et al.*, 1993). These data suggest that the very late genes compete for transcription factors, but that the p10 promoter requires at least one additional factor not involved in polyhedrin gene expression. This hypothesis contrasts with the observation that the relative activity of the very late promoters is not reduced by duplication of either the polyhedrin or the p10 promoter (Emery and Bishop, 1987; Weyer *et al.*, 1990).

Very late baculovirus promoters were analysed in detail by Zanotto *et al.* (1992) by comparing their DNA sequences. Since no consensus was observed in the genome region upstream of the 12-mer associated with the mRNA start site of polyhedrin and granulin genes (Rohrmann, 1986), their promoters might be internal as for class III genes as suggested by Zanotto *et al.* (1992). Class III genes are transcribed by RNA polymerase III and include tRNA and small 5S ribosomal RNA genes (Dillon, 1987; Lassar *et al.*, 1983). Early baculovirus genes (Fuchs *et al.*, 1983; Hoopes and Rohrmann, 1991) and host genes that encode proteins are transcribed by RNA polymerase II , while RNA polymerase I is involved in the production of the large ribosomal RNAs (Alberts *et al.*, 1983). Homologies to several structural elements of class III genes are present in the 5' leader sequence of baculovirus polyhedrin genes (Zanotto *et al.*, 1992). In the *Anticarsia gemmatalis* (Ag) MNPV polyhedrin gene, for instance, the TAAG element of the 12-mer is separated by two DNA helix turns from the motifs ATTGTA and TTTGTA, which are separated by one turn. In polyhedrin genes of other NPVs similar motifs are present (Fig. 7.1). The TAAG motif is associated with the mRNA start site. Deletions in the leader sequence of polyhedrin genes and insertions of linker molecules in this region, had a negative effect on the level of transcription (Possee and Howard, 1987; Ooi *et al.*, 1989). In known class III genes the TAAG motif is separated

from a TTAGTA motif by two helix turns. This may indicate that, like tRNA and 5S ribosomal RNA genes, baculovirus polyhedrin genes are transcribed by RNA polymerase III. The hyperactivity of these genes and the α-amanitin-resistant RNA polymerase activity present in baculovirus-infected cells (Huh and Weaver, 1990) further support this view. RNA polymerase III was previously shown to be involved in the expression of several adenovirus and Epstein-Barr virus late genes (Dillon, 1987).

In the p10 genes of AcMNPV (Kuzio *et al.*, 1984), *Bombyx mori* MNPV (Yaozhou, 1992), and *Spodoptera exigua* (Se) MNPV (Chapter 3) the TAAG motif and an A/TTTGTA motif are also present, although the second motif is not repeated as in polyhedrin genes. The the distance between these motifs is several nucleotides larger in the AcMNPV and BmNPV p10 genes. In the promoter region of the *Orgyia pseudotsugata* (Op) MNPV p10 gene (Leisy *et al.*, 1986) and *Choristoneura fumiferana* (Cf) MNPV (Wilson *et al.*, 1992), however, this second motif is absent. The relevance of the motif A/TTTGTA for p10 gene expression remains therefore unclear.

In general, baculovirus very late genes contain at least one poly (A) signal (AATAAA; Birnstiel *et al.*, 1985) in their trailer sequence (See Fig. 7.1). The length of this trailer sequence varies and can be upto 300 nucleotides in the case of the AcMNPV polyhedrin gene (Possee *et al.*, 1991). In the SeMNPV polyhedrin gene poly (A) signals are not present and polyadenylated mRNA could not be isolated (Van Strien *et al.*, 1992).

Very late gene expression is dependent on the expression of several early genes: The immediate early genes IE-1 and IE-n and the 'late essential factor' genes LEF-1, LEF-2 and LEF-3 (Passarelli and Miller, 1993a; 1993b; Li *et al.*, 1993). Sequence comparisons of these genes with other large DNA viruses and replication assays suggest that the LEF-genes as well as IE-1 and IE-n are essential for DNA replication (Kool, 1994) indicating an indirect effect

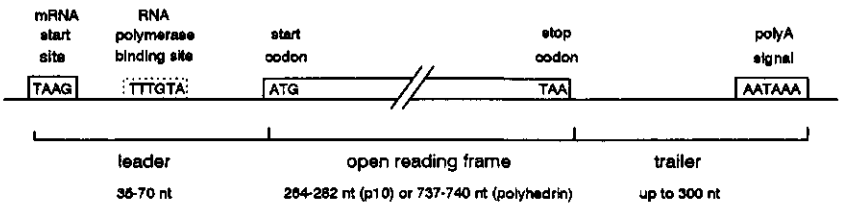

Figure 7.1. Schemetic representation of a typical baculovirus 'very late' gene showing the various structural elements (as boxes). The putative RNA polymerase binding motif (Zanotto *et al.*, 1992) is not present in all very late genes and is indicated by a dotted box.

on very late gene expression. Hence, the viral and host factors directly involved in very late gene expression remain to be identified. One strategy to identify the viral genes may be the isolation of baculovirus mutants affected in the expression of both polyhedrin and p10 genes, but not in the production of extracellular virus (ECV).

FUNCTIONAL ANALYSIS OF P10 PROTEINS

Early results

The product of the p10 gene should be characterized as a nonstructural protein, since it is not present in the two infectious forms of the virus, ECVs and PDVs. Immunofluorescence studies and immunogold labelling experiments have shown that p10 protein is associated with fibrillar structures formed in the nucleus and cytoplasm of cells infected with *Galleria mellonella* (Gm) MNPV, AcMNPV and OpMNPV (Croizier *et al.*, 1987; Quant-Russell *et al.*, 1987; Van der Wilk *et al.*, 1987). Polyhedrin is not present in these structures and mutants without an intact polyhedrin gene are still able to form fibrillar structures (Van der Wilk *et al.*, 1987). The fibrillar structures as seen in cross-sections of infected cells, at least those located in the nucleus, are probably all part of a continuous spiral-like structure as seen by confocal-laser scanning microscopy (D. Kasteel, unpublished results).

In early attempts to analyse the function of p10, the p10 gene has been disrupted by inserting a neomycine resistance gene (Croizier *et al.*, 1987; Gonnet and Devauchelle, 1987), or an enzymatic marker gene, such as lacZ (Vlak *et al.*, 1988; Williams *et al.*, 1989). These foreign gene insertions resulted in the disappearance of fibrillar structures (see also Table 7.1), indicating that p10 is an essential component of these structures. AcMNPV p10-lacZ fusion proteins, resulting from this mutagenesis, contained the amino-terminal 52 amino acids of p10 followed by the ß-galactosidase sequence and accumulated in granular structures in the nucleus of recombinant-infected cells (Vlak *et al.*, 1988; Williams *et al.*, 1989). Based on this result it was postulated (Fig. 7.2) that the amino-terminal 52 amino acids of p10 harboured a self-aggregation domain (Vlak *et al.*, 1988). Non-fused ß-galactosidase did not form such structures (Van Lent *et al.*, 1990).

Sequence analysis

With only the sequences of the closely related AcMNPV and OpMNPV p10 genes available, predictions of conserved amino acid sequences or distinct functional domains were difficult to make and might have been misleading. To obtain more reliable information, the p10 gene of the distantly related SeMNPV was characterized (Chapter 3). In contrast to AcMNPV and OpMNPV which can infect different insect hosts, SeMNPV is specific for *S. exigua*. The predicted p10 protein of SeMNPV had only minimal amino acid homology with the p10

proteins of AcMNPV and OpMNPV. During the course of the experimental research additional p10 protein sequences became available from other sources (see Chapter 5). Despite the diversity in nucleotide and amino acid sequence, p10 proteins have a similar size and a similar distribution of hydrophilic and hydrophobic amino acid residues. In particular the presence of a positively charged carboxy-terminus is notable, but also the presence of a hydrophobic domain in the amino-terminal segment appeares to be conserved.

Due to the low degree of nucleotide sequence homology, new p10 genes will be difficult to localize by hybridization with a known p10 gene as probe, especially when the viruses are distantly related. This problem can be circumvented by using cDNA derived from a 'very late' RNA preparation as a probe. This strategy was successful in the localization of the SeMNPV p10 gene (Chapter 3), but requires polyadenylated p10 mRNAs. The relative genetic location of all p10 genes known thus far is the same, in that it is situated between the p26 and p74 genes (see also Fig. 1.2). The p26 gene with unknown fuction is located upstream of p10 genes and is transcribed early in infection (Bicknell *et al.*, 1987). The p74 gene, a virulence gene for insects, is located downstream of p10 in an opposite transcriptional orientation and is essential for infectivity of polyhedra fro insects (Kuzio *et al.*, 1989). As alternative to exploiting the cDNA strategy, the p74 gene sequence may be used as a probe to find p10 genes as p74 genes appear to be much better conserved than p10 genes (Hill *et al.*, 1993; D. Zuidema, personal communication). To assign a protein as a p10 protein the carboxy-terminal part should contain several positively-charged amino acids, and the hydrophilicity distributions should give a similar profile as for known p10 proteins.

Fibrillar structures

The most remarkable similarity between p10 proteins is their positively charged carboxy terminus. To analyse the function of this domain further, a sequence of 8 or 15 amino acids was removed from the carboxy terminus of the AcMNPV p10 protein (Chapter 4). The truncated p10 polypeptides formed aggregates in the nucleus and cytoplasm of infected cells, but lacked the morphological features of wild-type fibrillar structures (See Table 7.1). Hence, the carboxy-terminal 8 amino acids ($RRGKRS_{92}S_{93}KK$) are essential for the assembly of fibrillar structures (Fig. 7.2). This positively charged sequence, which resembles the nuclear localization signal of polyhedrin (KRKK; Jarvis *et al.*, 1992), is apparently not involved in the intracellular distribution of p10. The protein is small enough to enter the nucleus by diffusion.

Monoclonal antibodies raised against the OpMNPV p10 protein showed a strong reaction with microtubules (MTs) in uninfected cells (Quant-Russell *et al.*, 1987). For AcMNPV association of p10 with microtubules has been reported by Volkman and Zaal (1990). These data suggested a MT cross-linking function for p10, but it is not necessary for

Table 7.1. Summary of the characteristics of AcMNPV p10 mutants described in this thesis and in literature[1]

Virus	encoded p10 protein	Additional modifications	P10 specific protein	P10 specific transcripts	P10 structures	Polyhedron release	Electron-dense spacers	Polyhedron envelope
AcMNPV	wild type	-	+	+	fibrillar	+	+	+
AcMO16	wild type	lacZ cassette	+	+	fibrillar	+	+	+
AcMO19	C-term Δ 8	lacZ cassette	+	+	aggregates	+	-	+
AcMO13	C-term Δ 15	lacZ cassette	+	+	aggregates	+	-	+
AcMO14	C-term Δ 42	lacZ cassette	not detected	+	not detected	-	+	-
AcAS3	none	lacZ cassette	-	-	-	-	+	-
AcMO21	none	Bsu36I site	-	-	-	not determined	likely	-
AcAK2	N-term 52 fused to ß-galactosidase	-	fusion protein	likely	granular	not determined	-	-
Ac229	N-term 52 fused to ß-galactosidase	-	fusion protein	likely	granular	-	+	-
Ac231	N-term 29 + 6 non-p10 amino acids	-	not detected	not determined	-	-	+	+
AcRK2	-	lacZ non-fused	-	-	-	-	+	+
AcCR1	SeMNPV p10	lacZ cassette	SeMNPV p10	likely	fibrillar	-	+	+
AcBA4	Ser_{92}→Ala_{92}	lacZ cassette	+	likely	fibrillar	+	+	+
AcBA5	Ser_{93}→Ala_{93}	lacZ cassette	+	likely	fibrillar	+	+	+
AcBA6	$Ser_{92,93}$→$Ala_{92,93}$	lacZ cassette	+	likely	fibrillar	+	+	+
VPN-neoR	GmNPV, N-term 52	phosphotrans-ferase gene	likely	likely	fibrillar	not determined	+	not determined

[1] Ac229 and Ac231 were described by Williams *et al.*, 1989; AcMO21 was described by Martens *et al.* (1994), AcAK2 by Vlak *et al.* (1988), and VPN-NeoR by Croizier *et al.*. (1987). LacZ cassette stands for a hsp70 promoter-lacZ-SV40 terminator gene cassette. N-term 52 stands for the 52 amino-terminal amino acids. C-term Δ 8 means a carboxy-terminal deletion of 8 amino acids. A p10 specific transcript means a transcript with homology to the AcMNPV p10 coding sequence. The term likely is used when the actual experiment is not performed, but when a positive results is predicted based on the characteristics of similar mutants.

the MT reorganization and depolymerization characteristic in a baculovirus infection (Volkman and Zaal, 1990). It is not clear, whether p10 located in the nucleus interacts with tubulin in the first place. In general, detectable amounts of tubulin are not present in cell nuclei. Whether tubulin is present in the nucleus of NPV-infected cells is unknown. The use of tubulin specific (monoclonal) antibodies would help addressing these questions.

The affinity of MT-associated proteins (MAPs) for microtubules is often regulated by phosphorylation of specific amino acids (Brugg and Matus, 1991; Steiner *et al.*, 1990). The carboxy-terminal sequence of AcMNPV p10 contains two serines and resembles known substrates for cAMP-dependent protein kinase (PKA) (Edelman *et al.*, 1987; Steiner *et al.*, 1990). The other known p10 proteins (Chapter 5) also contain at least one serine residue in their carboxy terminus, but not all of these serines fit into the consensus sequence for PKA substrates, RXXSX, where X can be any amino acid (Edelman *et al.*, 1987; Steiner *et al.*, 1990). The expression of the catalytic subunit of PKA by an AcMNPV recombinant resulted in phosphorylation of p10 at Ser_{92} or Ser_{93} (Cheley *et al.*, 1992). Biochemical experiments showed that this type of phosphorylation enhanced the affinity of p10 for microtubules (Cheley *et al.*, 1992). This PKA recombinant induced cell processes containing extended microtubules. The nature of the association of p10 within these structures is not known. Immunogold labelling may be used to study the interaction of p10 with MTs in these processes.

It can be envisaged that the aggregation of p10 is the first step in fibrillare structure formation. The transition of p10 from aggregates into fibrillar structures might require a certain degree of phosphorylated p10 in wild type AcMNPV-infected cells. To examine this possibility the serine residues in the carboxy terminus of p10 were replaced by alanines (Chapter 6). These modifications, however, did not affect the formation of fibrillar structures. The role of these serines in the formation of the cell processes, which were observed upon expression of PKA (Cheley *et al.*, 1992), can be further investigated by constructing recombinants that express both PKA and modified p10 proteins with Ala instead of Ser at position 92, 93 or both.

To study the specificity of fibrillar structure formation the distantly related p10 protein of SeMNPV was expressed by an AcMNPV recombinant that lacked its own p10 coding sequence (Chapter 5). SeMNPV p10 assembled into fibrillar structures in *S. frugiperda* cells in an otherwise AcMNPV context. These structures had the same morphological characteristics as upon infection of *S. exigua* cells with SeMNPV. Hence, there are no indications that other proteins, specific to the virus or to the host, are involved in this process. However, p10 proteins had a preference to form fibrils with p10 molecules of the same species, suggesting some specificity in aggregation and fibrillar structure formation.

A mutant that encoded only the amino-terminal 52 amino acids of the AcMNPV p10 protein was constructed (Chapter 4) to verify the results obtained with the p10-lacZ fusion mutants described by Vlak *et al.* (1988) and Williams *et al.* (1989), i.e. the self aggregation property. With this deletion mutant however, no detectable amounts of p10 were formed, although normal levels of p10-specific transcripts were present (see Table 7.1). Therefore, this mutant has not been analysed further. The analysis of p10-lacZ fusion mutants suggested the location of an aggregation domain in the amino-terminal half of p10 (Vlak *et al.*, 1988). This hypothesis is still valid as seen from the aggregation of p10 observed when 8 or 15 amino acids are removed from the carboxy terminus (Chapter 4).

Sequence comparisons using UWGCG programs revealed that p10 has amino acid sequence homology with lamins and other intermediate filament proteins (Van Oers, unpublished results). The amino-terminal half of p10 shows structural homology with the coil-1a region of mammalian lamins A and C, the *Drosophila* lamin Dm-0 (Gruenbaum *et al.*, 1988) and of other intermediate filaments (Fisher *et al.*, 1986; McKeon *et al*, 1986). Characteristic for these coil domains in intermediate filament proteins is a repeating pattern of seven amino acids, also known as the heptad repeat sequence, in which the first and fourth positions are occupied by hydrophobic amino acids or their polar substitutes. In the amino terminal half of p10 such a pattern is also present, although the heptad repeat domain in p10 is much smaller than in intermediate filaments, this homology may suggest, that p10 molecules, like intermediate filaments proteins, aggregate by coiled-coil interactions. In SeMNPV this heptad repeat structure is present in the entire p10 protein.

The fibrils of both nuclear and cytoplasmic fibrillar structures have a diameter of 5-6 nm (Hess *et al.*, 1989). Therefore, p10 protein is probably not an intermediate filament protein itself. Intermediate filaments have diameters of 8-10 nm. This size is intermediate to the other cytoskeletal filments: microfilaments (4-7 nm) and microtubules (25 nm) (Alberts *et al.*, 1983; Člampor, 1988). In addition, the size of p10 molecules is much smaller than of known intermediate proteins, which is 40-200 kDA.

Release of polyhedra from infected cells

Insect cells infected with p10 recombinants with either in- or out of-frame lacZ insertions failed to release polyhedra at the end of infection (Williams *et al.*, 1989). It was hypothesized that p10 might have a function in cell lysis. A p10 mutant lacking the entire p10 coding sequence (AcAS3, see Table 7.1) did not release polyhedra (Chapter 4) and confirmed the observation of Williams *et al.*, 1989). An enzymatic assay was used to determine whether the disruption of the cytoplasmic membrane was blocked in cells infected with a p10 deletion mutant (Chapter 4). With this assay it was shown that p10 is not involved in cell lysis, since the cytoplasmic membrane is disrupted irrespective of the presence

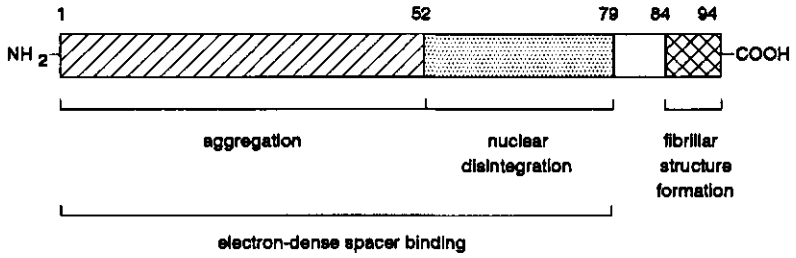

Figure 7.2. Model of AcMNPV p10 protein indicating the various functional domains. The numbers indicate amino acid positions.

of p10. Instead, p10 appears to be involved in the disintegration of the nuclear matrix, thereby releasing the polyhedra. The property of p10 to induce nuclear disintegration can be visualized by light microscopy and can be used as a simple criterium to discriminate wild type AcMNPV virus from p10 recombinants.

The p10 mutants, that produced truncated p10 proteins lacking the carboxy-terminal 8 or 15 amino acids (Chapter 4) and were unable to form fibrillar structures, were perfectly capable of releasing polyhedra from infected cells. This indicates that the fibrillar structure itself is not required for nuclear disintegration. May be this structure is transient in this process and causes nuclear breakdown upon dissassembly. Nuclear breakdown also occurs in the normal cell cycle during mitosis and is accompanied by the depolymerization of lamin proteins (see for review Newport and Forbes, 1987). In uninfected cells the S-phase precedes mitosis and baculoviruses appear to force infected-insect cells into this phase by for instance the ETL-gene product, which is homologous to rat proliferating cell nuclear antigen (O'Reilly *et al.*, 1989). The possible interaction of p10 with the proteins of the nuclear matrix, such as lamin proteins, should be investigated further. Wilson and Price (1987) have isolated nuclear matrices of infected insect cells and examined biochemically which proteins were associated with this structure. Unfortunately, however, they used ^{35}S-methionine to label viral proteins, a method inadequate to detect p10 protein, since this amino acid is not present in any known p10 protein. The experiment may be repeated with ^{3}H-leucine as a label or with specific antisera to detect p10 in the nuclear matrix.

The domain involved in nuclear disintegration has not yet been determined precisely. The p10-lacZ fusion mutants did not release polyhedra, indicating that this domain is not

located in the amino-terminal 52 amino acids. However, the ß-galactosidase part in this fusion protein may have hindered the functioning of the amino terminal 52 amino acids of p10. The region downstream of amino acid 79 is not involved, since a deletion of 15 amino acids is allowed without disturbing this function. The present hypothesis is that the nuclear disintegration domain is located between amino acid 52 and 79 (Fig. 7.2), but it may extend further towards the amino terminus than position 52.

Recently, a smaller p10 coding sequence was reported for a certain BmNPV strain (Hu *et al.*, 1993). The AcMNPV and BmNVP p10 proteins are highly similar (Chapter 5) and this BmNPV strain can be considered as a natural p10 deletion mutant that lacks the 24 carboxy-terminal amino acids. Based upon our results, the truncated BmNPV p10 protein will be unable to make fibrillar structures, since it lacks the carboxy terminus. It would be of interest to know whether this truncated p10 can disintegrate infected-cell nuclei, since this knowledge would allow us to map the disintegration domain more accurately.

An AcMNPV p26-p10 fusion mutant described by Chaabihi *et al.* (1993) was reported to induce pre-mature breakdown of infected cells. The fusion protein consisted of the 43 C-terminal amino acids of p10 fused to the 50 amino-terminal amino acids of p26, and was expressed behind the delayed-early p26 promoter. The fusion protein may cause premature disintegration since the nuclear breakdown domain of p10 is earlier expressed than in a wild type infection.

Cells infected with SeMNPV also released their polyhedra at the final stage of infection (Chapter 5). The SeMNPV p10 protein, however, could not induce nuclear disintegration when expressed in place of the AcMNPV p10 protein, irrespective of the type of host cell used. This result indicates that at least one additional viral protein may be involved in the process of nuclear disintegration, which only functions in combination with p10 from the homologous virus. The strategy adopted for the fine mapping of the nuclear disintegration domain of p10 includes the construction of AcMNPV recombinants expressing chimeric p10 proteins containing both AcMNPV and SeMNPV p10 sequences. The idea is that the AcMNPV p10 region that can rescue the nuclear disintegration function of SeMNPV p10 will harbour the disintegration domain. To identify the other viral factor(s) involved in nuclear disintegration, rescue experiments might be useful in which cells infected with the AcMNPV/SeMNPV-p10 recombinant are transfected with SeMNPV-cosmids or plasmids containing various parts of the SeMNPV-genome. Whether this method will prove succesful, is likely to depend on the efficiency of the transfection.

For granulosis viruses (GVs, *Baculoviridae;* Francki *et al.*, 1991) p10-like proteins have not yet been reported, but fibrillar structures are known from *Trichoplusia ni* GV-infected cells (Summers and Arnott, 1969). In cells infected with a GV the nucleus disintegrates earlier in the infection cycle than with NPVs (Tanada and Hess, 1991, for

review). A highly expressed late mRNA of a size in correspondence with a p10 messenger, is present in GV-infected cells and this transcript appears earlier than the granulin gene transcript (D. Winstanley, personal communication). If this mRNA is indeed the transcript of the granulosis virus p10 gene and if the function of the p10 gene product is similar in GVs, its earlier expression may explain the accelerated degradation of the nuclear structure. To answer this question, copy DNA may be used to map the gene and sequence analysis should reveal whether the transcript encodes a protein that matches the criteria for a p10 protein: its size, hydrophilicity profile and positively-charged carboxy terminus.

P10 deletion mutants are similar to wild type viruses in infectivity and virulence (Vlak *et al.*, 1989). The results described in this thesis indicate, that recombinant NPVs, in which the p10 promoter in its authentic locus is used for foreign gene expression, will release their polyhedra less efficient into the environment than wild type NPVs. Whether this biological containment is sufficient to clear the insect population from recombinant viruses after several generations has not been determined yet. The utilization of recombinants with a duplicated p10 promoter, leaving the original p10 locus intact (see for instance Weyer *et al.*, 1990), appears to be less favourable considering its biosafety aspects.

Polyhedron envelopes and electron-dense spacers

In nuclear polyhedrosis viruses the polyhedra are surrounded by an electron-dense envelope or calyx. This envelope mainly consists of carbohydrate (Minion *et al.*, 1979), but also contains a phosphorylated protein of approximately 34 kDa (pp34) in AcMNPV (Whitt and Manning, 1988). This protein is also present in so-called electron-dense spacers, present in the nucleus of infected cells (Zuidema *et al*, 1989; Van Lent *et al.*, 1990). The often close association of fibrillar structures with these electron-dense spacers and with polyhedron envelopes suggest a role for p10 in the formation of these structures.

Further evidence for the role of p10 in polyhedron morphogenesis came from the AcMNPV p10-lacZ fusion mutants described by Vlak *et al.* (1988) and Williams *et al.* (1989). The polyhedra of these fusion mutants were not surrounded by a polyhedron envelope. In one case electron-dense spacers were also absent (vlak *et al.*, 1988). A p10 deletion mutant, encoding the 29 amino-terminal amino acids of p10, did assemble electron-dense spacers and polyhedron envelopes, although a p10 protein could not be detected (Williams *et al.*, 1989). The polyhedron envelopes in this mutant were more loosely-fitting than in wild type AcMNPV. The difference in structure between the polyhedra of p10 deletion and p10-lacZ fusion mutants may be due to the presence of the amino-terminal 52 amino acids of p10, to the ß-galactosidase moiety or to the combination of both in the latter. It is however unlikely that ß-galactosidase is responsible, since a mutant expressing non-fused ß-galactosidase did induce spacers and polyhedron envelopes (Table 7.1; Van Lent *et al.*,

1990). The analysis of p10 mutant viruses with small deletions or point mutations may be more convenient to study the role of p10 in polyhedron envelope morphogenesis.

The p10 deletion mutants described in this thesis all formed polyhedron envelopes (Chapter 4; Table 7), indicating that p10 is not necessary for the formation of polyhedron envelopes. When 8 or 15 amino acids were deleted from the carboxy terminus of p10 electron-dense spacers were absent. This may suggest that the carboxy terminus has a second function. However, when p10 was completely absent, electron-dense spacers were present again (Chapter 4). These results, in combination with the frequently observed association of p10 with electron-dense spacers and polyhedron envelopes, suggest a possible binding site for pp34 on p10 molecules. The current point of view is that truncated p10 molecules that do not associate in fibrillar structures, but form aggregates (like the carboxy-terminal Δ 8 and Δ 15 mutants), expose too many binding sites, thereby dispersing pp34 over the entire aggregates and preventing the formation of electron-dense spacers. Immunogold labelling could reveal whether pp34 is indeed present in aggregates of these truncated p10 molecules. The putative binding site (Fig. 7.2) is located in the amino-terminal 79 amino acids and probably in the amino-terminal 52 amino acids, the part present in the p10-lacZ fusion mutants (Vlak *et al.*, 1988; Williams *et al.*, 1989). The binding site for AcMNPV pp34 is probably also present in the SeMNPV p10 protein, since this protein was able to interact with AcMNPV electron-dense spacers (Chapter 5).

The deletion mutants lacking 8 or 15 amino acids from the carboxy terminus did form polyhedron envelopes, although electron-dense spacers were absent (Chapter 4). This result demonstrates that spacers are not necessary as precursors for polyhedron envelopes, but are redundant envelopes.

Fibrillar structures in OpMNPV-infected cells reacted strongly at their periphery with antiserum against the OpMNPV polyhedron envelope protein (32 kDa protein) (Quant-Russell and Rohrmann, 1990; Quant-Russell *et al.*, 1991). Recently, Gross *et al.* (1994) reported that in OpMNPV both p10 and the 32 kDa protein are necessary to obtain an intimately sealed polyhedron envelope. In contrast to AcMNPV pp34, the OpMNPV 32 kDa protein is not required for polyhedron envelope formation, suggesting that the adhesion of carbohydrate to polyhedrin, may be sufficient to form a polyhedron envelope in OpMNPV. Such an adhesion of carbohydrate and polyhedrin from the inside was also reported for AcMNPV (Whitt and Manning, 1988). Without p10 or 32 kDa protein, however, 'holes' are present in the polyhedra of OpMNPV, the imprints of lost virions. Scanning electron microscopy could reveal whether such holes exist in the polyhedra of our AcMNPV deletion mutants. The isolated polyhedra of OpMNPV 32 kDa protein-minus/ p10-minus mutants aggregate. This is also the case for the AcMNPV pp34-minus mutant (J.M. Vlak, personal communication).

The function of the polyhedron envelope therefore seems to be twofold: to seal of polyhedra to prevent loss of virions and to avoid aggregation of polyhedra (Gross *et al.*, 1994).

Final remarks

P10 is a 'very late' viral protein that is made in copious amounts in NPV-infected cells. Although p10 proteins of different NPV species show a high degree of amino acid sequence variation, the main characteristic of these proteins is that they assemble into fibrillar structures in the nucleus and the cytoplasm of infected cells. Therefore, the name fibrillin appears to be an appropriate name for this protein.

The major function of p10 seems to be the breakdown of infected cell nuclei, resulting in the efficient dissemination of polyhedra into the environment. This function of p10 is independent of fibrillar structure formation and is NPV specific. Based on the analysis of p10 mutant viruses a model is proposed (Fig. 7.2) for the location of several distinct functional domains on the AcMNPV p10 protein: (i) amino acids 1 to 52 harbour a domain for intermolecular interaction of p10 molecules and are probably also involved in binding polyhedron envelopes and electron-dense spacers, (ii) the sequence from 52 to 79 contains amino acids involved in nuclear disintegration, (iii) amino acids 87 to 94 are required for fibrillar structures formation and are possibly involved in interaction with tubulin. P10 is not required for the formation of polyhedron envelopes and spacers. Results obtained in this study have extended our insight in the structure and function of baculovirus p10 proteins. The role of p10 in conjunction with the polyhedron envelope protein in the intimate sealing of remains somewhat enigmatic.

REFERENCES

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1983). *Molecular Biology of the Cell*, Garland Publishing, New York.

Bicknell, J.N., Leisy, D.J., Rohrmann, G.F. and G.S. Beaudreau (1987). Comparison of the p26 gene region of two baculoviruses. *Virology* 161: 589-592.

Birnstiel, M.L., Busslinger, M. and Strub, K. (1985). Transcription termination and 3' processing. The end is in site! *Cell* 41: 349-359.

Brugg, B., and Matus, A. (1991). Phosphorylation determines the binding of microtubule-associated protein 2 (MAP2) to microtubules in living cells. *Journal of Cell Biology* 114 : 735-743.

Chaabihi, H., Ogliastro, M.H., Martin, M., Giraud, C., Devauchelle, G. and Cerutti, M. (1993). Competition between baculovirus polyhedrin and p10 gene expression during infection of insect cells. *Journal of Virology* 67: 2664-2671.

Cheley, S., Kosik, K.S., Paskevich, P., Bakalis, S. and Bayley, H. (1992). Phosphorylated baculovirus p10 is a heat stable microtubule-associated protein associated with process formation in SF-9 cells. *Journal of Cell Science* 102: 739-752.

Člampor, F. (1988) The role of cytoskeleton and nuclear matrix in virus replication. *Acta Virologica* 32: 168-189.

Croizier, G., Gonnet, P. and Devauchelle, G. (1987). Localisation cytologique de la protéin p10 du baculovirus de la polyédrose nucléaire du Lépidoptère *Galleria mellonella*. *C.R. Acad. Sci. Paris. t. 305*, Serie III: 677-681.

Dillon, L.S. (1987). The 5S ribosomal and other small RNAs. In *The gene: Structure, Function and Evolution*. pp 91-143. New York, Plenum Press.

Edelman, A.M., Blumenthal, D.K., and Krebs, E.G. (1987). Protein serine/threonine kinases. *Annual Review of Biochemistry* 56: 567-613.

Emery, V.C. and Bishop, D.H.L. (1987). The development of multiple expression vectors for high level synthesis of eukaryotic proteins: expression of LCMV-N and AcNPV polyhedrin protein by a recombinant baculovirus. *Protein Engineering* 1: 359-366.

Fisher, D.Z., Chaudhary, N. and Blobel, G. (1986). cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. *Proceedings of the National Academy of Sciences USA* 83: 6450-6454.

Francki, R.I.B., Fauquet, C.M., Knudson, D. and Brown, F. (eds) (1991). Classification and nomenclature of viruses. Fifth Report of the International Committee on Taxonomy of Viruses. *Archives of Virology* supplementum II: pp 450.

Friesen, P.D. and Miller, L.K. (1986). The regulation of baculovirus gene expression. In : *The Molecular Biology of Baculoviridae* (W. Doerfler and P. Böhm, eds). *Currenty Topics in Microbiology and Immunology* 131: 31-49.

Fuchs, L.Y., Woods, M.S. and Weaver, R.F. (1983). Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection: A novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. *Journal of Virology* 48: 641:646.

Gonnet, P. and Devauchelle, G. (1987). Obtention par recombinaison dans le gène du polypeptide p10 d'un baculovirus exprimant le gène de résistance à la néomiycine dans les cellules d'insecte. *C.R. Acad. Sci. Paris. t. 305*, Serie III: 111-114.

Gross, C.H., Russell, R.L.Q. and Rohrmann, G.F. (1994). The *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: 000-000.

Gruenbaum, Y., Landesman, Y., Drees, B., Bare, J.W., Saumweber, H., Paddy, M.R., Sedat, J.W., Smith, D.E., Benton, B.M. and Fisher, P.A. (1988). *Drosophila* nuclear lamin precursor Dm-0 is translated from either or two developmentally regulated mRNA species apperently encoded by a single gene. *Journal of Cell Biology* 106: 585-596.

Hess, R.T., Goldsmith, , P.A. and Volkman, L.E. (1989). Effect of cytochalasin D on cell morphology and AcMNPV replication in a *Spodoptera frugiperda* cell line. *Journal of Invertebrate Pathology* 53: 169-182.

Hill, J.E., Kuzio, J., Wilson, J.A., MacKinnon, E.A. and Faulkner, P. (1993). Nucleotide sequence of the p74 gene of a baculovirus pathogenic to the spruce budworm, *Choristoneura fumiferana* multicapsid nuclear polyhedrosis virus. *Biochimica et Biophysica Acta* 1172: 187-189.

Hoopes, R.R. and Rohrmann, R.F. (1991). *In vitro* transcription of baculovirus immediate early genes: Accurate mRNA initiation by nuclear extracts from both insect and mammalian cells. *Proceedings of the national Academy of sciences USA* 88: 4513-4517.

Hu, N.E. and Weaver, R.F. (1990). Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus. *Journal of Invertebrate Pathology* 12: 83-85.

Hu, N.T.T., Lu, Y.F. and Hou, R.F. (1993). *Bombyx mori* nuclear polyhedrosis virus p10 gene, complete coding sequence. GenBank Accession number L13071.

Jarvis, D.L., Bohlmeyer, D.A. and Garcia, Jr. A. (1991). Requirements for nuclear localization and supramolecular assembly of a baculovirus polyhedrin protein. *Virology* 185: 795-818.

Kool, M. (1994). *Baculovirus DNA Replication*. PhD thesis, Agricultural University Wageningen, The Netherlands.

Kuzio, J., Rohel, D.Z., Curruy, C.J., Krebs, A., Carstens, E.H. and Faulkner, P. (1984). Nucleotide sequence of the p10 polypeptide gene of *Autographa californica* nuclear polyhedrosis virus. Virology 139: 414-418.

Kuzio, J., Jaques, R. and Faulkner, P. (1989). Identification of p74, a gene essential for virulence of baculovirus occlusion bodies. *Virology* 179: 759-763.

Lasser, A.B., Martin, P.L. and Roeder, R.G. (1983). Transcription of class III genes: formation of preinitiation complexes. *Science* 222: 740-748.

Leisy, D.J., Rohrmann, G.F. and Beaudreau, G.S. (1986). Nucleotide sequence and transcriptional mapping of the *Orgyia pseudotsugata* nuclear polyhedrosis virus p10 gene. *Virology* 153: 157-167.

Li, Y., Passarelli, A.L. and Miller, L.K. (1993). Identification, sequence, and transcriptional mapping of lef-3, a baculovirus gene involved in late and very late gene expression. *Journal of Virology* 67: 5260-5268.

Martens, J.W.M., Van Oers, M.M., Van de Bilt, B., Vlak, J.M. and Oudshoorn, P. (1994). Efficient recovery of baculovirus recombinants expressing foreign genes under p10 promoter control. Submitted.

McKeon, F.D., Kirschner, M.W. and Caput, D. (1986). Homologies in both primary and secondary structure between nuclear envelope and intermediate filament proteins. *Nature* (London) 319: 463-468.

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

Newport, J. W. and Forbes, D.J. (1987). The nucleus: structure, function, and dynamics. *Annual Review of Biochemistry* 56: 535-565.

Ooi, B.G., Rankin, C. and Miller, L.K. (1989). Downstream sequences augment transcription from the essential initiation site of a baculovirus polyhedrin gene. *Journal of Molecular Biology* 210: 721-736.

O'Reilly, D.R., Crawford, A.M. and Miller, L.K. (1989). Viral proliferating cell nuclear antigen. *Nature* (London) 337: 606.

Passarelli, A.L. and Miller, L.K. (1993a). Three baculovirus genes involved in late and very late gene expression: IE-1, IE-n and lef-2. *Journal of Virology* 67: 2149-2158.

Passarelli, A.L. and Miller, L.K. (1993b). Identification and characterization of lef-1, a baculovirus gene involved in late and very late gene expression. *Journal of Virology* 67: 3481-3488.

Possee, R.D. and Howard, S.C. (1987). Analysis of the polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus. *Nuclei Acids Research* 15: 10233-10248.

Possee, R.D., Sun, T.-P., Howard, S.C., Ayres, M.D., Hill-Perkins, M. and Gearing, K.L. (1991). Nucleotide sequence of the *Autographa californica* nuclear polyhedrosis virus 9.4 kbp *Eco*RI-I and -R (polyhedrin gene) region. *Virology* 185: 229-241.

Quant-Russell, R.L., Pearson, M.N., Rohrmann, G.F. and Beaudreau, G.S. (1987). Characterization of baculovirus p10 synthesis using monoclonal antibodies. *Virology* 160: 9-19.

Quant-Russell, R.L. and Rohrmann, G.F. (1990). A baculovirus polyhedron envelope protein: Immunogold localization in infected cells and mature polyhedra. *Virology* 174: 177-184.

Quant-Russell, R.L., Pearson, M.N. and Rohrmann, G.F. (1991). Immunoelectron microscopic examination of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus-infected *Lymantria dispar* cells: time course and localization of major-associated proteins. *Journal of General Virology* 72: 275-283.

Roelvink, P.W., Van Meer, M.M.M., De Kort, C.A.D., Possee, R.D., Hammock, B.D. and Vlak, J.M. (1992). Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and p10 genes. *Journal of General Virology* 73: 1481-1489.

Rohrmann, 1986. Polyhedrin structure. *Journal of General Virology* 67: 1499-1513.

Smith , G.E., Summers, M.D. and Fraser, M.J. (1983). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Molecular Cell Biology* 3: 2156-2165.

Steiner, B., Mandelkow, E.M., Biernat, J., Gustke, N., Meyer, H.E., Schmidt, B., Mieskes, G., Sölling, H.D., Drechsel, D., Kirschner, M.W., Goedert, M. and Mandelkow, E. (1990). Phosphorylation of microtubule-associated protein tau: identification of the site for Ca^{2+} -calmodulin dependent kinase and relationship with tau phosphorylation in Alzheimer tangles. *The EMBO Journal* 9: 3539-3544.

Summers, M.D. and Arnott, J.J. (1969). Ultrastructural studies on inclusion formation and virus occlusion in nuclear polyhedrosis virus and granulosis virus-infected *Trichoplusia ni* (Hübner). *Journal of Ultrastructural Research* 28: 462-480.

Tanada, Y. and Hess, (1991). Baculoviruses. Granulosis virus. In: *Atlas of Invertebrate Viruses* (J.R. Adams and J.R. Bonami, eds). CRC-Press, Boca Raton, Florida, USA, pp 227-257.

Van der Wilk, F., Van Lent, J.W.M. and Vlak, J.M. (1987). Immunogold detection of polyhedrin, p10 and virion antigens in *Autographa californica* nuclear polyhedrosis virus-infected *Spodoptera frugiperda* cells. *Journal of General Virology* 68: 2615-2623.

Van Lent, J.W.M., Groenen, J.T.M., Klinge-Roode, E.C., Rohrmann, G.F., Zuidema, D. and 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 Strien, E.A., Zuidema, D., Golbach, R.W. and 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.

Vlak, J.M., Smith, G.E. and Summers, M.D. (1981). Hybridization selection and *in vitro* translation of *Autographa californica* nuclear polyhedrosis virus mRNA. *Journal of Virology* 40: 762-771.

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klinge-Roode, E.C., Geervliet, J.B.F., Roosien, J. and Van Lent, J.W.M. (1988). Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-ß-galactosidase fusion gene. *Journal of General Virology* 69: 765-776.

Vlak, J.M. (1993). Genetic engineering of baculoviruses for insect control. In: *Molecular Approaches to Fundamental and Applied Entomology* (J. Oakeshott and M.J. Whitten, eds), pp. 90-127. Springer-Verlag, New York.

Volkman, L.E. and Zaal, K.J.M. (1990). *Autographa californica* nuclear polyhedrosis virus : Microtubules and replication. *Virology* 175: 292-302.

Weyer, U., Knight, S. and Possee, R.D. (1990). Analysis of very late gene expression by *Autographa californica* nuclear polyhedrosis virus and the further development of multiple expression vectors. *Journal of General Virology* 68:2615-2623.

Whitt, M.A. and 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.

Williams, G.V., Rohel, D.Z., Kuzio, J. and Faulkner, P. (1989). A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *Journal of General Virology* 70: 187-202.

Wilson, J.A., Hill, J.E., Kuzio, J. and Faulkner, P. (1992). Nucleotide sequence of *Choristoneura fumiferana* nuclear polyhedrosis virus p10 gene (unpublished data). GenBank, Accession number M98513.

Wilson, M.E. and Price, K.H. (1988). Association of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) with the nuclear matrix. *Virology* 167: 233-241.

Yaozhou, Z. (1992). Studies on the p10 gene of *Bombyx mori* nuclear polyhedrosis virus. Chinese Academy of Agricultural Sciences, PhD. thesis. Graduate School of Chinese Academy of Agricultural Sciences, Shanghai Institute of Biochemistry, Academica Sinica.

Zanotto, P. M. de, Sampaio, M.J.A., Johnson, D.W., Rocha, T.L. and Maruniak, J.E. (1992). The *Anticarsia gemmatalis* nuclear polyhedrosis virus polyhedrin gene region: sequence analysis, gene product and structural comparisons. *Journal of General Virology* 73:1049-1056.

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

SUMMARY

Nuclear polyhedrosis viruses belong to the family *Baculoviridae* and cause fatal diseases in arthropods predominantly in insects of the order *Lepidoptera*. These viruses were first reported in silk worms where viral infections could have devastating effects on the production of silk. In nature these viruses regulate the size of insect populations. Each nuclear polyhedrosis virus can only infect a small number of host insects and these viruses are harmless for mammals and birds. Therefore, these viruses are used as biological insecticides. The interest in these insect viruses was further augmented by the observation that they could be used for the high level expression of foreign genes.

The exploitation of nuclear polyhedrosis viruses requires understanding of their biology and molecular organization. Infected insect cells produce two proteins in large quantities in the late phase of infection: polyhedrin (approximately 30 kDa), the matrix protein of the viral occlusion bodies, and a protein of 10 kDa (p10). This p10 protein is not present in virions, but is associated with fibrillar structures observed in the nucleus and cytoplasm of infected cells. The study described in this thesis focused on the functional analysis of the p10 protein.

Polyhedrin and p10 genes have promoters with a similar structure and hence, a similar regulation mechanism was expected. Both promoters might compete for transcription factors, resulting in reduced expression levels of each individual gene. The effect of the simultaneous activity of the p10 and polyhedrin promoter on the level of p10 gene expression was examined by analysing *Autographa californica* (Ac) MNPV recombinants with various deletions in the polyhedrin gene (Chapter 2). None of the deletions in the polyhedrin gene resulted in higher expression from the p10 promoter and no significant difference in level of expression was observed between the p10 and polyhedrin promoter. The results suggest that, the p10 and polyhedrin gene, despite the homology in their promoters, are independently regulated.

At the beginning of this research only the amino acid sequence of the p10 proteins of the closely related AcMNPV and *Orgyia pseudotsugata* (Op) MNPV were available. To benefit from amino acid sequence data for the prediction of conserved domains, the p10 gene of the distantly related baculovirus *Spodoptera exigua* (Se) MNPV was characterized (Chapter 3). The deduced SeMNPV p10 protein showed limited amino acid sequence identity to the p10 proteins of OpMNPV and AcMNPV (39% and 26%, respectively). The three p10 proteins, however, had similar hydrophilicity profiles and most notably, they all had a positively-charged carboxy terminal sequence.

On the basis of sequence comparisons, AcMNPV p10 deletion mutants were constructed to identify putative functional domains (Chapter 4). Several domains were found in the p10 protein : (i) The hydrophilic carboxy terminus (position 87 to 94) is required for the assembly of p10 into fibrillar structures. Without this stretch of amino acids p10 molecules form aggregates that lack the morphological features of fibrillar structures. This domain might be involved in the interaction with tubulin. (ii) Amino acids 1-52 have a self-aggregation fucntion (see also Vlak *et al.*, 1988; *J. Gen. Virol.* 69: 765-776). (iii) P10 is involved in the disintegration of infected-cell nuclei, which leads to the release of individual polyhedra. This function is located in the amino-terminal 79 amino acids, and is independent of fibrillar structure formation. It is likely that these 79 amino acids also harbour a binding site for electron-dense spacers and polyhedron envelopes.

The p10 protein of SeMNPV shows only limited homology to the p10 protein of AcMNPV (Chapter 3), although it probably has the same functions. To investigate the functional homology between these two p10 proteins, a recombinant AcMNPV virus was constructed, which encoded the SeMNPV p10 protein instead of the authentic p10 protein (Chapter 5). In an AcMNPV environment the SeMNPV p10 protein assembled into fibrillar structures in *S. frugiperda* cells, and is therefore functionally homologous to AcMNPV p10. These structures had the same morphology as in *Spodoptera exigua* cells infected with SeMNPV. P10 has a preference to aggregate with p10 molecules of the same species, as was concluded from simultaneous expression of SeMNPV and AcMNPV p10. The SeMNPV p10 protein was not able to induce nuclear disintegration, when expressed from the AcMNPV genome, regardless of the type of host cells used. This indicates that at least one other viral protein is involved in this process, which specifically recognizes p10 of the same species.

The carboxy-terminal amino acid sequence (RRGKRSSK), involved in the formation of fibrillar structures, contains two serine residues, which are prone to phosphorylation, for instance by cAMP-dependent protein kinase (Cheley *et al.*, 1992. *J. Cell. Sci.* 102: 739-752). We hypothesized that aggregates of p10 might form fibrillar structures due to the presence of a small fraction of phosphorylated p10 molecules. To test this hypothesis one or both serines were replaced with alanines (Chapter 6). These p10 mutants normally induced fibrillar structures. Phosphorylation on these serines is therefore not involved in this process.

The disintegration of infected-cell nuclei at the end of the infection seems to be the major function of p10 proteins. Due to this disintegration polyhedra are released as individual particles, resulting in an efficient dissemination of progeny virus into the environment. Wild-type viruses may therefore have a selective advantage over genetically-modified viruses with altered p10 genes, when these modified viruses are used in biological control.

SAMENVATTING

Kernpolyedervirussen behoren tot de familie *Baculoviridae* en veroorzaken een fatale ziekte bij geleedpotigen (*Arthropoda*), voornamelijk bij insekten van de orde *Lepidoptera*. Deze virussen werden het eerst ontdekt in de zijderupscultuur, waar virusinfecties enorme economische schade aan konden richten. In de natuur reguleren deze virussen de omvang van insektenpopulaties. Iedere soort kernpolyedervirus kan slechts een beperkt aantal gastheren infecteren en deze virussen zijn niet infectieus voor vogels en zoogdieren. Daarom worden ze door de mens ingezet voor specifieke bestrijding van plaag-insekten. De interesse in deze groep van insektevirussen nam verder toe, nadat bleek, dat ze benut konden worden om heterologe genen tot expressie te brengen.

Om deze virussen met succes te kunnen gebruiken is gedegen kennis van hun biologie en moleculaire organisatie een eerste vereiste. Geïnfecteerde insektecellen produceren laat in infectie twee typen eiwitten in grote hoeveelheden: het polyhedrine eiwit (ongeveer 30 kDa), dat de virusdeeltjes beschermt tegen afbraak, en een eiwit vam ongeveer 10 kDa (p10). Dit p10 eiwit komt niet voor in virusdeeltjes, maar in fibrillaire structuren in de kern en het cytoplasma van geïnfecteerde cellen. Het onderzoek beschreven in dit proefschrift was er op gericht om de functie van dit p10 eiwit op te helderen.

De genen voor polyhedrine en p10 lijken qua structuur van hun promoters sterk op elkaar en eenzelfde regulatiemechanisme werd daarom verondersteld. Dit zou kunnen betekenen dat de promoters elkaars transcriptie factoren wegvangen. Om dit te onderzoeken werden mutanten gemaakt van het kernpolyedervirus van *Autographa californica* (AcMNPV) met deleties in het polyhedrine gen (Hoofdstuk 2). De expressie vanaf de p10 promoter werd niet gehinderd door de aanwezigheid van een functioneel polyhedrine gen. Dit is een sterke aanwijzing, dat deze twee genen ondanks hun gelijksoortige promoter onafhankelijk gereguleerd worden. Significante verschillen in activiteit tussen de p10 en polyhedrine promoter werden niet gevonden.

Bij de start van het onderzoek waren slechts de p10 genen van twee kernpolyedervirussen gekarakteriseerd. Om aan de hand van de aminozuurvolgorde van de gecodeerde p10 eiwitten voorspellingen te kunnen doen ten aanzien van geconserveerde sequenties en functionele domeinen, werd een derde p10 gen gekarakteriseerd (Hoofdstuk 3) en wel van het kernpolyedervirus van *Spodoptera exigua* (SeMNPV). De overeenkomst in de aminozuurvolgorde tussen de drie p10 eiwitten bleek gering. Wel waren de drie p10 eiwitten ongeveer even groot en vertoonden ze grote overeenkomst in de manier waarop geladen en ongeladen

aminozuren over het eiwit waren verdeeld. Inmiddels zijn de sequenties van de p10 eiwitten van meer kernpolyedervriussen bekend en opvallend is dat deze alle een sterk positief geladen sequentie aan de carboxy-terminus bezitten (Hoofdstuk 5).

Op grond van de vergelijking van p10 eiwitten werden deleties aangebracht in het p10 gen van AcMNPV. Op deze manier werden een aantal functionele domeinen geïdentificeerd in het p10 eiwit (Hoofdstuk 4). De positief geladen carboxy-terminus (aminozuur 87 tot 94) is essentieel voor de vorming van fibrillaire structuren. De amino-terminale helft (aminozuur 1 tot 52) heeft een aggregatie functie (zie ook Vlak *et al.*, 1988; *J. Gen. Virol.* 69: 765-776). Het bleek, dat p10 is betrokken bij de degradatie van celkernen aan het einde van de infectie, waardoor polyeders, die de nieuwe virusdeeltjes bevatten, vrijkomen. Deze functie ligt binnen aminozuur 1-79 en is onafhankelijk van de vorming van fibrillaire structuren.

Het p10 eiwit van SeMNPV vertoont slechts geringe homologie met het p10 eiwit van AcMNPV (Hoofdstuk 3), ondanks het feit dat het waarschijnlijk dezelfde functies heeft in het infectie-proces. Om de functionele homologie tussen deze p10 eiwitten te onderzoeken werd een recombinant AcMNPV virus gemaakt, dat in plaats van het authentieke p10 eiwit, het p10 eiwit van SeMNPV tot expressie bracht (Hoofdstuk 5). Het SeMNPV p10 eiwit was in staat om in een AcMNPV omgeving in *S. frugiperda* cellen fibrillaire structuren te vormen en is dus functioneel homoloog met AcMNPV p10. Deze structuren hadden dezelfde morfologie als in *Spodoptera exigua* cellen na een infectie met SeMNPV. Bij gelijktijdige expressie van SeMNPV en AcMNPV p10 bleek een voorkeur tot aggregatie met p10 moleculen van dezelfde soort. In een AcMNPV omgeving kon het SeMNPV p10 eiwit echter geen kerndegradatie bewerkstelligen ongeacht de gebruikte gastheercellen. Dit geeft aan dat er bij dit proces nog een ander viraal eiwit betrokken is, dat alleen het eigen p10 eiwit herkent.

De carboxy-terminale aminozuur sequentie (RRGKRSSK), die betrokken is bij de vorming van fibrillaire structuren, bevat twee serine-residuen die gevoelig zijn voor fosforylering, bijvoorbeeld door cAMP afhankelijk kinase (Cheley *et al.*, 1992. *J. Cell. Sci.* 102: 739-752). Aggregaten van p10 zouden daarom via incidentele fosforylering aan deze serines in staat kunnen zijn om fibrillaire structuren te vormen. Om deze hypothese te toetsen werden een of beide serines vervangen door alanines (Hoofdstuk 6). Deze p10 mutanten vormden echter normale fibrillaire structuren. De fosforylering van p10 aan deze serines speelt dus geen rol in dit proces.

De desintegratie van celkernen aan het einde van de infectie lijkt de belangrijkste functie van p10 eiwitten te zijn. Hierdoor komen de polyeders als afzonderlijke deeltjes vrij, hetgeen resulteert in een efficiënte verspreiding van het virus in de natuur. Wild-type virussen zouden hierdoor een selectief voordeel kunnen hebben ten opzichte van genetisch gemodificeerde virussen met veranderde p10 genen, wanneer deze laatste gebruikt worden bij biologische bestrijding van insekten.

LIST OF ABBREVIATIONS

AcMNPV	*Autographa californica* nuclear polyhedrosis virus
ß-gal	ß-galactosidase
BmNPV	*Bombyx mori* nuclear polyhedrosis virus
bp	basepair
BSA	bovine serum albumin
CfMNPV	*Choristoneura fumiferana* nuclear polyhedrosis virus
ECV	extracellular virus
EDTA	ethylene diamine tetra-acetic acid disodium salt
FBS	fetal bovine serum
hr-region	highly repetitive region
hsp	heat shock promoter
kbp	kilobase pair
kDa	kilodalton
LDH	lactate dehydrogenase
NAD^+	nicotine-amide adenine dinucleotide
ORF	open reading frame
NPV	nuclear polyhedrosis virus
MNPV	multiple-nucleocapsid nuclear polyhedrosis virus
m.o.i.	multiplicity of infection
MT	microtubule
m.u.	map unit
NOB	non-occluded baculovirus
nt	nucleotide
ONP	2-nitrophenol
ONPG	2-nitrophenyl-ß-D-galactopyranoside
OpMNPV	*Orgyia pseudotsugata* nuclear polyhedrosis virus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDV	polyhedron- derived virion
PE	polyhedron envelope

p.i.	post infection
PKA	cAMP-dependent protein kinase
PnMNPV	*Perina nuda* nuclear polyhedrosis virus
SeMNPV	*Spodoptera frugiperda* nuclear polyhedrosis virus
SSC	buffer containing sodium chloride and sodium citrate
SDS	sodium dodecyl-sulphate
SNPV	single-nucleocapsid nuclear polyhedrosis virus
SV40	simian virus 40
TBS	Tris buffered saline
TCID	tissue culture infective dose
Tris	2-amino-2-(hydroxymethyl)-1,2-propandiol
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

LIST OF PUBLICATIONS

Van Oers, M.M., Malarme, D., Jore, J.M.P. and Vlak, J.M. (1992). Expression of the *Autographa californica* nuclear polyhedrosis virus p10 gene: effect of polyhedrin gene expression. *Archives of Virology* 123: 1-11.

Van Oers, M.M., Flipsen, J.T.M., Reusken, C.B.E.M., Sliwinsky, E.L., Goldbach, R.W. and Vlak, J.M. (1993). Functional domains of the p10 protein of *Autographa californica* nuclear polyhedrosis virus. *Journal of General Virology* 74: 563-574.

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

Van Oers, M.M., Flipsen, J.T.M., Reusken, C.B.E.M. and Vlak, J.M. (1994). Specificity of p10 functions. *Virology* 200: 000-000.

Martens, J.W.M., Van Oers, M.M., Van de Bilt, B., Vlak, J.M. and Oudshoorn, P. (1994). A novel baculovirus vector that facilitates the generation of p10-based recombinants. Submitted.

CURRICULUM VITAE

Monique Maria van Oers werd geboren op 9 oktober 1962 te Amersfoort. In 1981 behaalde zij het Gymnasium diploma (ongedeeld) aan het Stedelijk Gymnasium te Utrecht, waarna ze Biologie ging studeren aan de Rijksuniversiteit van Utrecht. In 1984 werd het kandidaatsexamen behaald. In 1989 studeerde zij af met als hoofdvak Moleculaire Genetica (Dr. S.A. Langeveld en Prof. Dr. P.J. Weisbeek) en de bijvakken Electronenmicroscopische Structuuranalyse (Dr. W. Linnemans) en Moleculaire Biologie (Prof. Dr. H.O. Voorma). Van mei 1989 tot mei 1992 was zij als wetenschappelijk onderzoeker verbonden aan de Landbouw Universiteit te Wageningen op een derdegeldstroom project gefinancierd door Solvay & Cie, Brussel, België. Van mei 1992 tot september 1993 genoot zij een deeltijd-aanstelling, die het mogelijk maakte het onderzoek beschreven in dit proefschrift af te ronden. Vanaf maart 1994 werkt zij bij de werkgroep Plantevirussen van de vakgroep Biochemie van de Rijksuniversiteit van Leiden o.l.v. van Prof. Dr. J.F. Bol.