THE MOLECULAR CHARACTERISATION OF A BACULOVIRUS ISOLATED FROM *TRICHOPLUSIA NI.*

by

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submitted in partial fulfillment of the requirements for the degree of Philosophiae Doctor in the Department of Microbiology University of the Western Cape.

Promoter:

Professor S. Davison

2001

DECLARATION

I declare that "The genomic characterisation of a baculovirus isolated from T*richoplusia ni*" is my own work and that all the sources I have used or qouted have been indicated and acknowledged by means of complete references.

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Burtram Clinton Fielding

15 MARCH 2001

Date

BIOGRAPHICAL SKETCH

Burtram Clinton Fielding was born on the 07 February 1973 in Cape Town, South Africa. He attended various primary schools amongst which Buckingham Primary School and Accordion Primary School. In 1990 he matriculated from Symphony Senior Secondary School, Belhar. Burtram enrolled at the University of Stellenbosch for a MB.ChB. in 1991. In 1992 he enrolled at the University of the Western Cape and obtained a B.Sc. degree in 1994. He completed a B.Sc. Hons degree (*cum laude*) in Microbiology in 1995 at the same university. In 1996 he enrolled for a Master's degree, which was ungraded in 1998 to a P.hD. degree at the University of the Western Cape. Burtram was awarded M.Sc. and P.hD. FRD Prestigious Equity Scholarships during 1997 and 1999, respectively. He has been appointed as Lecturer in the Department of Microbiology, at the University of The Western Cape, since January 2001.

LIST OF PUBLICATIONS

1. Fielding B.C. and Davison S. 1999. The characterisation and phylogenetic relationship of the *Trichoplusia ni* single capsid nucleopolyhedrovirus polyhedrin gene. *Virus Genes* 19:1, 67-73 (**Chapter 3**).

2. Fielding B.C. and Davison S. 2000. Identification and characterisation of the *Trichoplusia ni* single capsid nucleopolyhedrovirus *p10* gene. *Virus Genes* 20:2, 189-192 (**Chapter 4**).

3. Fielding B.C., Khan S., Wang W. and Davison S. 2000. The characterisation of three baculoviruses with potential as biological control agents - Review. *The South African Journal of Science*. Submitted (**Chapter 1**).

4. Wang W., Leat N., Fielding B.C. and Davison S. 2001. Identification, sequence analysis and phylogeny of the immediate early gene 1 of the *Trichoplusia ni* single nucleocapsid polyhedrosis virus. *Virus Genes*. In Press.

5. Fielding B.C. and Davison S. The genetic organization of a 2966bp DNA fragment of a single capsid nucleopolyhedrovirus isolated from *Trichoplusia ni.* In preparation for "Virus Research". (**Chapter 5**)

6. Fielding B.C. and Davison S. 2000. Functional and physical genomic map of a single capsid nucleopolyhedrovirus isolated from ***Trichoplusia ni.*** In preparation for "Virus Research". (**Chapter 2**)

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♥This thesis is dedicated to Portia and Ezra♥

PREFACE

This thesis is presented as a compilation of five chapters, with a complete reference list at the end of the thesis. All the chapters are written in the same format and each chapter is introduced separately. Chapter 3 and Chapter 4, which are written according to the format of *Virus Genes,* have been published. Chapter 1 has been submitted as a review article to the *South African Journal of Science*. Chapter 2 and Chapter 5 are written as research articles for *Virus Research.*

Chapter 1 **Literature Review and Research Rational**
The molecular characterisation of a baculovirus isolated from *Trichoplusia ni*

Chapter 2 **Research Results**
The identification and characterisation of a baculovirus isolated from a field population of *Trichoplusia ni*

Chapter 3 **Research Results**
The characterisation and phylogenetic relationship of the *Trichoplusia ni* single capsid nucleopolyhedrovirus polyhedrin gene

Chapter 4 **Research Results**
Identification and characterisation of the *Trichoplusia ni* single capsid nucleopolyhedrovirus *p10* gene

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Summary

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
et al.	And others
nm	Nanometer
µm	Micrometer
µl	Microliter
IPM	Integrated pest management
Tni	*Trichoplusia ni*
Bt	*Bacillus thuringiensis*
ds	Double stranded
ss	Single stranded
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
NPV	Nucleopolyhedrovirus
GV	Granulovirus
MNPV	Multi-capsid NPV
SNPV	Single-capsid NPV
hpi	Hours post infection
PDV	Polyhedra derived virus
BV	Budded virus
kDa	Kilodalton

°C	Degree Celsius
kbs	Kilobases
i.e.	That is
ORF	Open reading frame
OB	Occlusion bodies
NaAc	Sodium Acetate
Na_2CO_3	Sodium Carbonate
dH_2O	Distilled water
m.u.	Map units
REN	Restriction endonuclease
U	Units
PCR	Polymerase chain reaction

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Chapter 1

Literature Review and Research Rationale

The molecular characterisation of a baculovirus isolated from *Trichoplusia ni*

A portion of the work presented in this chapter has been submitted for publication as: **The characterisation of three baculoviruses with potential as biological control agents.** ***The South African Journal of Science.***

Chapter 1

1.1 Introduction

"About 10, 000 species of the more than 1 million species of insects are crop-eating, and of these, approximately 700 species worldwide cause most of the insect damage to man's crops, in the field and in storage."

Synthetic chemical pesticide manufacture and usage escalated dramatically in the period of technological advancement during and following the Second World War. The vast majority of chemical insecticides currently in use are long-lasting compounds that affect the nervous systems of insects on contact. It has been demonstrated, however, that these nonselective compounds are often harmful not only to the target insects, but also other beneficial organisms including the pests' natural parasitoids and predators. The environmental and social costs of pesticide use in the U.S. have been estimated to be at least $1 billion each year. Due the growing awareness of the environmental damage and relative ineffectiveness of repeatedly used synthetic chemical pesticides, viable safer alternatives of pest control are being sought. Integrated pest management (IPM) is a technology developed for pest management with the aim of achieving desired control levels while reducing the use of chemical pesticides. In order for IPM to be successful, various combinations of chemical, biological and physical controls are utilized. With IPM both the pest and beneficial non-target organisms are monitored to determine whether pest numbers actually warrant treatment. A properly and extensively implemented IPM system could reduce pesticide usage by as much as 50%, without compromising the quality of pest control (http://www.funkandwagnalls.com/encyclopedia).

Trichoplusia ni (Hübner: *Noctuidae*) is a polyphagous crop pest distributed throughout Africa, North America from Canada to Mexico, also in Hawaii, Europe and Asia. Cabbage looper (*common name*) larvae have been implicated in the damage of the crucifer family, cabbage, spinach, sugarbeets, peas, celery, potatoes, alfalfa, beans, tomato, certain ornamental plants and mint, too name but a few. Larvae feed between veins on the underside of lower leaves, with larger larvae chewing ragged holes in the foliage (Figure 1.1). Although *Trichoplusia ni* (*T. ni*) has successfully been controlled with synthetic chemical pesticides, increased resistance to these insecticides have been reported (Dornan *et al.*, 1995). Similarly, the combined use of traditional pheromone releasers and blacklight traps has proven inadequate in crop protection (Debolt *et al.*, 1979). *Bacillus thuringiensis* has been used in the effective control of cabbage loopers, but resistance to Bt toxin used for long periods has been reported. Thus, the need for an effective long-term control agent is rather urgent.

Eight families of insect viruses are recognised, consisting of more than 650 viruses, differing in morphological, biophysical and biochemical characteristics (Table 1.1). Usually the larval stage is infected, resulting either in acute or inapparent infections, the former ultimately resulting in insect death. The vast majority of known viruses infect members from the order Lepidoptera (Ignoffo, 1979). Three of the virus families contain members that are occluded within a protein matrix. This proteinaceous occlusion body, which is characteristic of cytoplasmic polyhedrosis viruses, entomopoxviruses and baculoviruses, is unique to invertebrate viruses (Tweeten *et al.*, 1981). With the exception of the baculoviruses, members of the different insect virus families closely resemble vertebrate viruses in morphology and biochemical characteristics (Consigli *et al.*, 1983; Tweeten *et al.*, 1981).

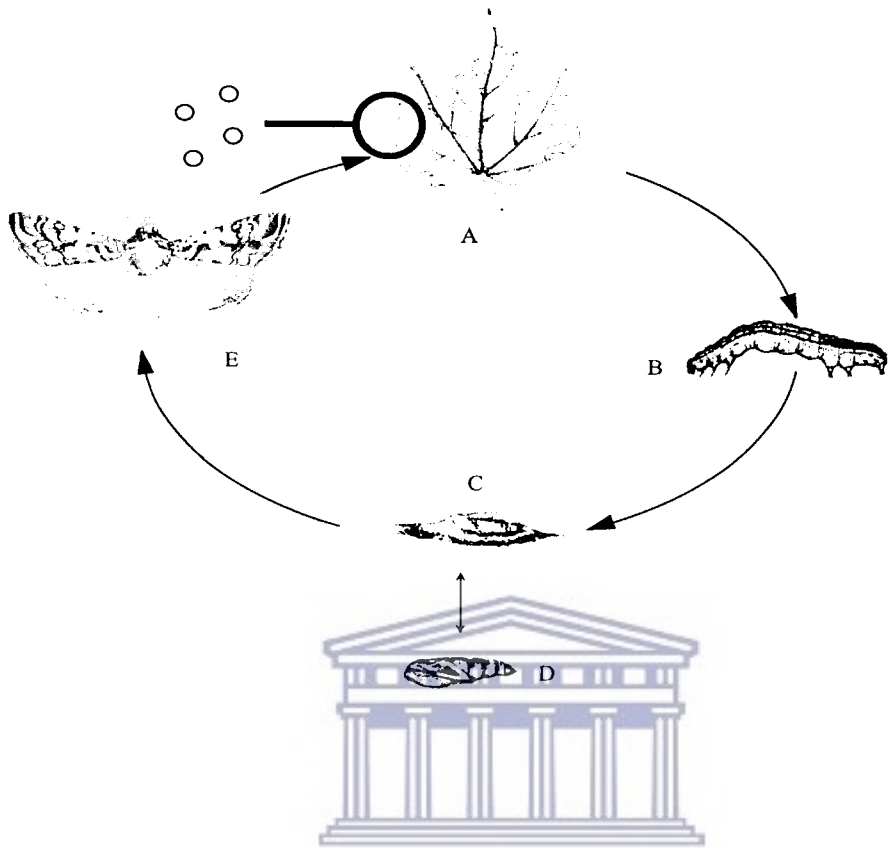


Figure 1.1: Typical life cycle of *Trichoplusia ni*. The average generation time of Tni is between 26-40 days. This can be influenced by various factors, including temperature, diet and length of day-night cycle. (A) Following mating, females deposit between 200-300 greenish-white eggs on the upper or lower surfaces of upper canopy leaves. Usually this occurs over a 10-12 day period. Eggs hatch within two to three days. (B) The pale green larvae feed actively for about 14-21 days, (C) after which they spin flimsy silken cocoons and pupate. (D) Initially pupae are yellowish-green, but darken to brown before the adult moth emerges nine days later. (E) The semi-nocturnal moth is smoky, grayish-brown in colour and the forewings are marked with a distinctive figure 8-shaped, silver-white spot. Mature moths feed on plant nectar and can live for up to 24 days (Adlerz, 1971; Kishaba *et al.*, 1976; Marsden, 1979; Hofmann and Frodsham, 1993).

Table 1.1: Classification of viral families infecting invertebrates (Consigli *et al.*, 1983).

Virus Family	**Type of nucleic acid**	**Example**
Reoviridae	dsRNA	Cytoplasmic polyhedrovirus†
Rhabdoviridae	ssRNA	Sigmavirus
Togaviridae	ssRNA	Arbovirus
Parvoviridae	ssDNA	Densovirus
Picornaviridae	ssRNA	Nudaurelia capensis Virus
Poxviridae	dsDNA	Entomopoxvirus†
Iridoviridae	dsDNA	Iridovirus
Baculoviridae	dsDNA	Granulovirus†
		Nucleopolyhedrovirus†

†members are occluded within a protein matrix

ds, Double stranded; ss, Single stranded.

Baculoviruses appear to be the most promising from a biological control perspective. The Family *Baculoviridae* are invertebrate-specific pathogens that contain genomes consisting of a single circular supercoiled, double stranded DNA molecule ranging in size from 80-220 kilobases (Kool *et al.*, 1995; Miller, 1988; Arif, 1986). Traditionally, baculoviruses have been classified into Subgroups A, B and C (Matthews, 1982), however, this has been superseded by a revised classification scheme (Francki *et al.*, 1991). The family consists of two genera, the nucleopolyhedrovirus (NPVs) and the granulovirus (GVs). Furthermore, the NPV genus has been subdivided into the multiple nucleocapsid per envelope (MNPV) and the single nucleocapsid per envelope (SNPV) non-phylogenetic groups. Of the more than

600 viruses found in the family, only nineteen have received species status. These include 15 assigned NPVs and 4 GVs (Murphy *et al.*, 1995).

1.2 Granuloviruses

Granuloviruses have been isolated from about 150 insect species (Jehle and Backhaus, 1994), all within the order Lepidoptera (Blissard and Rohrman, 1990; Tweeten *et al.*, 1981). GVs infecting different Lepidoptera are structurally complex and exhibit wide variations in host specificity, serological properties as well as biochemical characteristics (Tweeten *et al.*, 1980). Nonetheless, all are very similar in morphological characteristics (Stairs *et al.*, 1966).

Usually GVs consist of a single virion embedded within an occlusion body (Kang *et al.*, 1997). Occasionally, however, GVs can consist of multiple enveloped nucleocapsids per occlusion body (Arnott and Smith, 1968; Smith *et al.*, 1990; Crook and Brown, 1982). Each nucleocapsid, with average dimensions of 30-60nm by 260-360nm, is surrounded by an envelope. Furthermore, each enveloped nucleocapsid is embedded within an ellipsoidal protein matrix composed of granulin, forming a structure approximately 300 to 500nm in length (Rohrmann, 1992) by 120 to 350nm in width (Tweeten *et al.*, 1981).

Studying the effect of a GV infection on *Heliothis armigera* (Hübner), Whitlock (1974) described various symptoms common to GV infections. He noted irregular larval growth rates commencing 4 - 6 days post infection, with infected insects remaining in the larval stage for a longer-than-normal period of time and growing larger than normal. Moreover, a gradual lightening of the cuticle was observed,

with the larvae appearing almost white upon death. Simultaneously, the integument took on a shiny, almost translucent appearance. Mortality always followed the onset of the 4th instar, mostly just prior to pupation and the integument of the dead insect remained intact and sturdy.

1.3 Nucleopolyhedroviruses

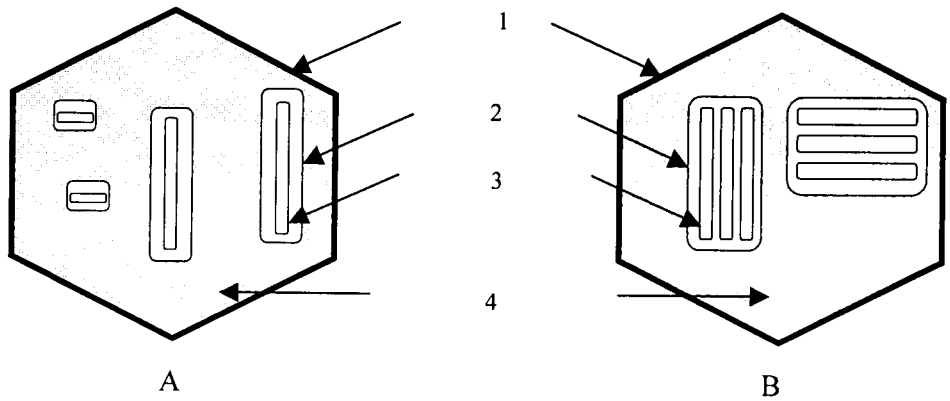
Nucleopolyhedroviruses infect in excess of 600 insect species, with a single virus isolate normally restricted to one host or a few related species (Blissard and Rohrmann, 1990). Although some members of the genus NPV infect crustaceans, the majority infect insects (Washburn *et al.*, 1999). Whereas SNPVs have been isolated from *Hymenoptera, Lepidoptera, Diptera, Coleoptera, Trichoptera* and *Siphonoptera* (Kool *et al.,* 1995; King and Possee, 1994), MNPVs have only been isolated from the evolutionary youngest insect order, the *Lepidoptera* (Washburn *et al.*, 1999). This would suggest that MNPVs originated from the SNPVs and then radiated along with their hosts through evolutionary time (Rohrmann, 1986). Polyhedra range in size from about 0.5 µm to 15 µm, with the predominant size range between 0.6 µm to 2.5 µm. The rod-shaped nucleocapsids with dimensions of approximately 40nm - 60nm by 250nm - 300nm, may range in number from 1 to 29 per virion (Adams and McClintock, 1991).

Normally symptoms are not visible for several days following NPV ingestion, with behavioural changes usually the first indication of metabolic disturbances (King and Possee, 1994). Typically, the infected larvae exhibit a loss in appetite, which is characterised by the subsequent cessation of feeding and migration away from the food source. This abnormal migratory behaviour has been well documented for baculovirus infected *Lymantria manacha* and *Neodiprion swainei,* the former migrating to the top of

the plant where they die, hanging by their prolegs (Smirnoff, 1965). Death occurs with liquefaction of the internal tissues and darkening of the cuticle, often resulting in the bursting of the epidermis, liberating masses of infectious viral occlusion bodies (Bonning and Hammock, 1996). This leads to contamination of surrounding vegetation and the virus is subsequently spread by wind, birds and insects over wide areas (Rohrmann, 1992).

1.4 Pathogenicity

Depending on the viral strain, baculoviruses are slow to kill their hosts, requiring from 3 to 5 days, for the more virulent strains (Kunimi *et al.*, 1996) to a few months (Crook, 1996 personal communication) to kill the infected host. They need to infect a succession of susceptible hosts to insure the maintenance of the virus population. The occluded virus that can overwinter on plant surfaces or in the soil, enters the larvae primarily *per os*. Secondary routes of entry have been proposed by Kelly (1982), including spiracles, artificial wounds or abrasions and parasitism, but these are unlikely to play a major role in infection, since Berghold (1953) demonstrated that occluded viruses introduced directly into the haemolymph is non-infective. Following ingestion by a susceptible host, the protein matrix is solubilised by the alkaline contents of the insect midgut (Berghold, 1947; Faust and Adams, 1966) and the released nucleocapsids enter the midgut columnar epithelial cells by fusion with the microvilli (Bonning and Hammock, 1996). Viral enhancement factors within the occlusion bodies trigger specific biochemical and structural changes in the epithelial peritrophic membrane, thereby facilitating viral entry (Derksen and Granados, 1988).



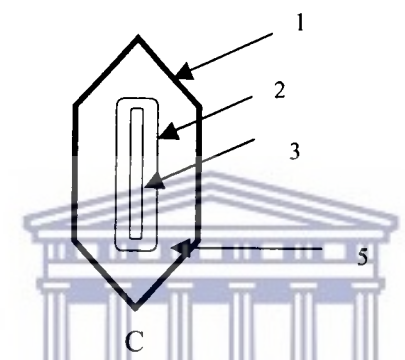


Figure 1.2: Schematic representation of the different genera in the Family *Baculoviridae*. A: SNPV, B: MNPV and C: GV. The GV is represented by a single virion per nucleocapsid, although multiple virions per nucleocapsid have been observed. 1: Polyhedral envelope protein; 2: Virion envelope; 3: Capsid protein; 4: Polyhedrin; 5: Granulin (Blissard and Rohrmann, 1990).

1.5 Baculovirus replication *in vivo*

The nucleocapsids are transported to the nucleus (in the case of NPV) or the cytoplasm (in the case of GV), where the viral DNA uncoats, genes are expressed and DNA replicates (Blissard and Rohrmann,

1990). As yet, it is unclear whether the nucleocapsids inject the DNA into the nucleus via a nuclear pore or whether the entire nucleocapsid enters the nucleus (King and Possee, 1994), the latter being widely accepted (Washburn *et al*., 1999; Granados, 1978). In the case of NPVs, the virus undergoes a primary round of replication and progeny nucleocapsids can be observed as early as 8 hours post infection (h.p.i.). Histopathologically, at this stage, the cell exhibits an enlarged nucleus and virogenic stroma are observed within the nucleus (Blissard and Rohrman, 1990).

Progeny nucleocapsids assembled within and around the dense virogenic stroma could produce one of two distinct virion phenotypes in the biphasic replication cycle (Blissard and Rorhman, 1990; Kelly, 1982): those virions occluded within polyhedra are termed "polyhedra-derived virus" (PDV) and those found in the haemocoel of infected insects, is known as "budded virus" (BV) (King and Possee, 1994; Blissard and Rohrman, 1990; Kelly, 1982). Some nucleocapsids bud through the nuclear membrane and are transported to the cytoplasm, where they lose their nuclear-derived envelope. These naked nucleocapsids bud through the cytoplasmic membrane into the haemolymph, acquiring the budded virus specific envelope. Other progeny nucleocapsids become enveloped within the nucleus by an envelope synthesized *de novo*. Subsequently, they are occluded within polyhedrin protein that crystallizes around them and a polyhedral envelope forms around periphery of occluded virions (King and Possee, 1994; Blissard and Rohrman, 1990).

Although PDV and BV are genetically identical (King and Possee, 1994; Blissard and Rohrman, 1990), they differ in morphology and protein composition (Figure 1.3), source of virion envelopes and cell specificity (Blissard and Rohrman, 1990). Additionally, whereas BV is responsible for the

dissemination of the infection throughout the insect, infecting the fat bodies, nerve cells and haemocytes, the PDV is responsible for the horizontal transfer between insects (Bonning and Hammock, 1996; Blissard and Rohrman, 1990; Miller, 1988).

1.6 Baculovirus replication *in vitro*

The lack of susceptible insect cell lines has prevented the detailed study of GV gene expression and virus replication (Dwyer and Granados, 1987; Hashimoto *et al.*, 1996). NPVs, on the other hand, have a relatively wider host range, with AcMNPV infecting up to 25 different cell lines (Du and Thiem, 1997). Therefore, the vast majority of baculovirus knowledge has been gained through the study of AcMNPV infection of *Spodoptera frugiperda* cells. *In vitro* studies on AcMPNV have proven invaluable in elucidating fundamental knowledge of baculovirus DNA replication, gene function, expression and the regulation thereof. This cell-virus system offers the possibility to investigate the molecular basis of host specificity. Also, the availability of a susceptible cell line is imperative for both the construction and screening of recombinant viruses (Maeda, 1995). Although baculoviruses do not replicate in nonpermissive cell lines, they are able to enter and express certain genes (Thiem *et al.*, 1996).

1.7 DNA Replication

Cis- and *trans*-acting elements are involved in DNA replication (Kool *et al.*, 1994). The *cis*-acting elements include unique non-homologous regions (Lu *et al.*, 1997) and palindromic repeats in homologous regions (*hr*), dispersed on the baculovirus genome. Five *trans*-acting elements are involved in DNA replication (Chen *et al.*, 1999). These include DNA polymerase, DNA helicase, and

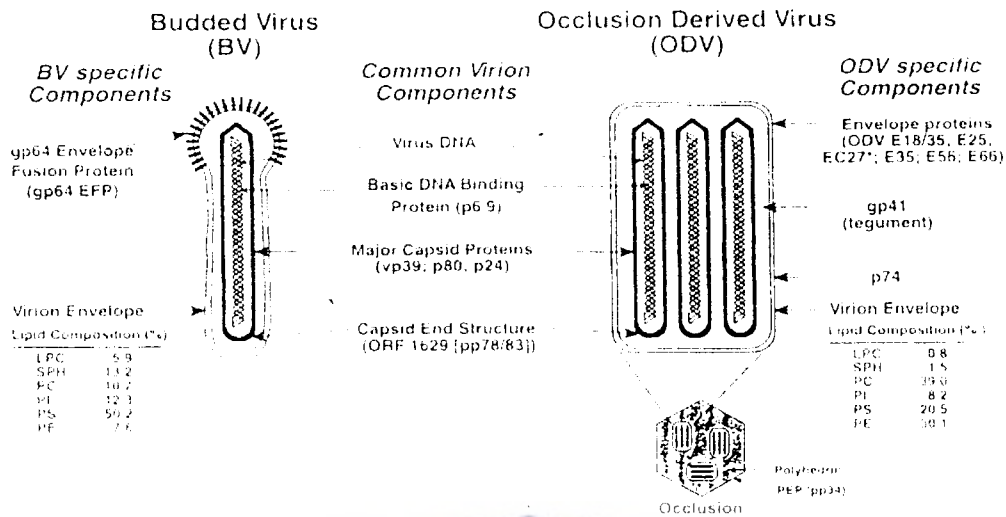


Figure 1.3: Structural components of the two NPV phenotypes generated during a typical infection cycle. Common and phenotypic-specific components are indicated. The BV phenotype is represented by a virion with a single nucleocapsid and the PDV is represented by an MNPV (from Blissard and Rohrmann, 1990).

three late expression factors (LEFs). The *lef* genes are thought to encode a primase (*lef1*), a primase-processivity factor *(lef2)* and a single-stranded DNA binding protein (*lef3*) (Chen *et al.*, 1999). *Lef2*, which has been identified in MNPVs (Ahrens and Rohrmann, 1995), SNPVs (Chen *et al.*, 1999) and GVs (Jehle *et al.*, 1997) has been shown to be essential for DNA replication (Lu and Miller, 1995) and

late gene expression (Merrington *et al.*, 1996).

1.7 Gene Expression

Baculovirus gene expression occurs in a transcriptional cascade (Kool *et al.*, 1995; Blissard and Rohrmann, 1990; Friessen and Miller, 1986), in which gene products of one temporal class of gene transactivate the transcription of genes in the next temporal class (Figure 4). Gene expression can been divided into two general categories, *i.e.* early gene expression and late gene expression. Whereas, early genes are transcribed by the host RNA polymerase, late genes are transcribed by a virus-specific RNA polymerase with a unique subunit composition (Yang *et al.*, 1991). Furthermore, early gene expression precedes viral DNA replication, with late gene expression occurring after or concurrently with the start of viral DNA replication (Blissard and Rohrmann, 1990; Friesen and Miller, 1986). Each phase consists of two functionally defined stages, including immediate early (IE) and delayed early (DE) gene expression, as well as late and very late gene expression (Friessen and Miller, 1986). Since IE genes can be transcribed either in the presence of protein inhibitors (Kelly and Lescott, 1981) or by uninfected insect cells (Blissard and Rohrmann, 1990), their activation is not dependant on the presence of other viral gene products (King and Possee, 1994). However, the presence of IE gene products is required for the activation of the DE genes.

AcMNPV with a genome size of 128 kbp, is capable of encoding at least 150 proteins (Frederici and Maddox, 1996; Kamita *et al.* 1993; Kool *et al.*, 1995). With the exception of the trans-activating factor gene *ie*-1, splicing does not seem to play a significant role in the regulation of most baculovirus genes (Rohrmann, 1992). Current evidence suggests that gene expression is not influenced by exact gene

location on the genome or their proximity to other specific genes (Rohrmann, 1992).

1.8 Latent baculovirus infections

Numerous apparently sporadic outbreaks of NPV infections in natural populations of insects have been reported (Longworth and Cunningham, 1968; Evans and Harrap, 1982; Campbell, 1981). These appeared to be due to the activation of a latent NPV. Latent virus is apparently activated by feeding insects heterologous NPVs (Hughes *et al.*, 1993; McKinley *et al.*, 1981; Ponsen and de Jong, 1964; Kelly *et al.*, 1981; Smith, 1963). It has also been suggested that various stress factors such as extreme temperatures, overcrowding, oral administration of various chemicals or the application of X-rays could activate latent viruses (Volkman, 1997).

Kukan (1999) suggested that baculovirus latency could either be due to the viral DNA integrating into the host genome as found in hepatitis B infection (Howard, 1986), or that the virus could be maintained as independent viral genetic material in the host cell nucleus as with herpes simplex (Mellerik and Fraser, 1987). There is some, although speculative evidence that these latent viruses exist as persistent infections (Hughes *et al.*, 1997), remaining at low levels of infection with the expression of viral genes. However, as yet, the exact mechanism of baculovirus latency is not completely understood.

1.9 Eukaryotic expression vectors

Initial interest in baculoviruses was stimulated because of their ability to over express heterologous eukaryotic genes under the control of baculovirus very late gene promoters (Smith *et al.*, 1983; Kool *et al.*, 1995). These proteins are conveniently processed in a manner similar to their native form (Kool *et*

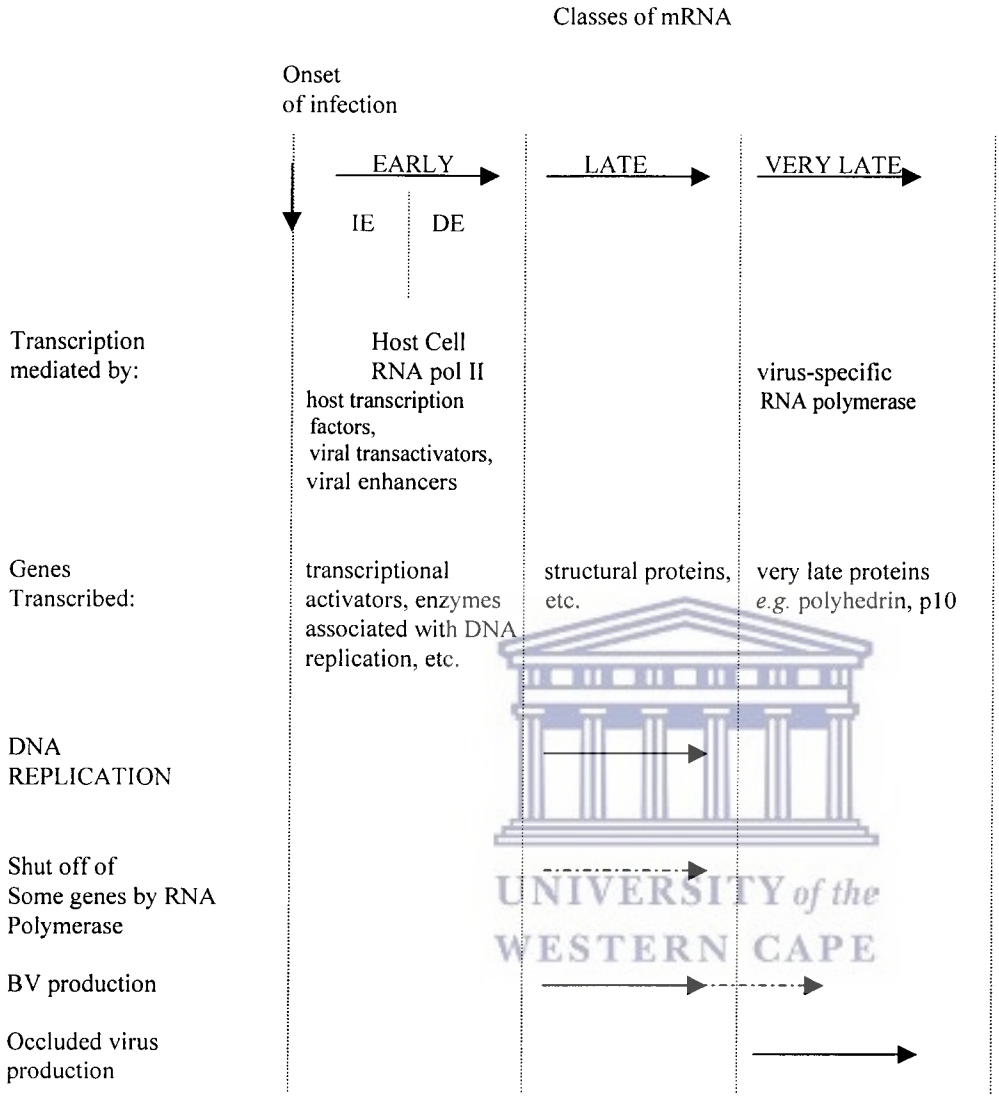


Figure 1.4: Representation of the transcriptional cascade of baculovirus gene expression. The relationships between early, late and very late gene transcription and DNA replication during a typical baculovirus infection cycle is indicated (Modified from Rohrmann, 1986; King and Possee, 1994). IE: Immediate early; DE: Delayed early

al., 1995). Recombinant proteins including cytosolic, nuclear, mitochondrial, membrane bound and secreted proteins are reliably expressed using baculovirus expression vectors in insect cell lines (Kost and Condreay, 1999). Recombinant baculovirus (RBVs) infected insect cells have been successfully used for the synthesis of functional multi-subunit protein structures. This system has also been invaluable for studying the viral particle assembly process, which is being used for the development of vaccine candidates based on the production of virus-like particles and conventional recombinant antigens (Newcomb *et al.*, 1996; Ke *et al.*, 1999). In particular, baculovirus very late gene promoters have been used in the commercial expression of various important proteins, amongst others the expression of human α-interferon using BmNPV (Maeda *et al.*, 1985), the human interleukin 2 gene (Smith *et al.*, 1985), the influenza virus haemagglutonin gene (Possee, 1986), as well as β-interferon (Smith *et al.*, 1983) and β-galactosidase (Pennock *et al.*, 1984) using AcMNPV.

1.10 Genetic modification of NPVs

To date, a few hundred baculoviruses have been isolated from various pest larvae, of which only about 40 are considered useful from a biocontrol point of view (Maeda, 1995). This can be mainly attributed to the lower efficacy of many baculoviruses compared to classical insecticides (Volkman, 1995). In fact, baculoviruses, depending on the strain, can require anything from about 3 days (Kunimi *et al.*, 1996) to about 3 months (Crook, personal communication) to kill the infected host. This problem could be remedied by recombinant DNA technology. Two approaches have been followed: 1) foreign genes encoding for insect-specific toxins or insect neurohormones , or hormone regulators, have been added to baculovirus genomes (Kunimi *et al.*, 1996; Miller, 1995) or 2) deletion of existing baculovirus genes (O'Reilly and Miller, 1991).

The insertion of insect-specific toxins or neurohormones *in lieu* of the polyhedrin gene, under the transcriptional control of the polyhedrin promoter, has resulted in varying degrees of success. Genetically modified baculoviruses expressing active scorpion toxins (Cory *et al.*, 1994; Steward *et al*, 1991), mite neurotoxins (Tomalski and Miller, 1991) and juvenile hormone esterase (Hammock *et al.*, 1990), have been more successful than those expressing *Bt* toxins (Pingel and Lewis, 1997; Merryweather *et al.*, 1990). Deletion of the *egt* gene (regulation of insect moulting pathway) from the baculovirus genome leads to decrease feeding time and premature death (Riegel *et al.*, 1994).

Baculoviruses are host-specific, with a single isolate usually infecting only one or a few closely related insect species. Although this makes them attractive from an environmental perspective, it is less so from a commercial one. Therefore, to increase the commercial attraction of baculoviruses as biopesticides, the host range needs to be broadened. Since host range is determined primarily by various genes, including helicase, *p35*, *iap*, *lef*-7, *hcf*-1 and *hrf*-1, genetic manipulation of these genes could result in broadening the host range of a particular virus.

1.11 Baculovirus Proteins

Since transfection of viral DNA into viable susceptible cultured insect cells leads to the production of complete infective virions, it is evident that under these conditions no virion-associated structural proteins are required for virus replication (Rohrmann, 1992). To date, only one PDV associated protein (p74) is known to be essential for successful infection of insects (Kuzio *et al.*, 1989). Certain baculovirus genes have been studied in detail, including polyhedrin, *p10* and the anti-apoptotic genes *iap* and *p35*.

Polyhedrin, a 29-kiloDalton protein is the primary constituent of the crystalline matrix surrounding the enveloped nucleocapsids (Gombart *et al.*, 1989; Weyer and Possee, 1989). It accounts for between 18% and 50% of the total alkali-soluble stainable protein present in infected cells (Blissard and Rohrmann, 1990); this occurs approximately 70 hours post infection (Miller, 1988). Polyhedrin is essential for the persistence of the biological activity of the virus in the environment. Non-occluded viruses have been found to be rapidly inactivated in the soil and on plant tissues as well as in dead larvae. Therefore, the major function of polyhedrin appears to be the stabilisation of the virions in the environment, protecting the viral DNA against solar UV radiation and protecting important structural components (Blissard and Rohrmann, 1990). Homology studies have identified polyhedrin as the most conserved baculovirus protein so far characterised. Since polyhedrin is not essential for the formation of infectious virions (Smith *et al*, 1983), its promoter is frequently utilised in baculovirus expression vector systems to drive heterologous transcription of foreign genes (McLachlin and Miller, 1994).

Similar to polyhedrin, *p10* is over expressed very late in the infection cycle. It is commonly found associated with polyhedra (Quant-Russell *et al.*, 1987) and it affects virus replication in several ways, with specific regions within the peptide shown to confer unique functions (Wilson *et al.*, 1995). *P10* forms part of the fibrillar structures observed in virus assembly (Van der Wilk *et al.*, 1987), leading to greater occlusion body stability and it plays an important role in calyx formation and attachment (Wilson *et al.*, 1995). Although *p10* has been conserved throughout baculovirus evolution, the primary amino acid sequence has not been well conserved. Nevertheless, evolutionary pressure has maintained certain secondary and tertiary structures, including a N-terminal coiled-coil structure that is involved in the intramolecular interaction leading to the aggregation of *p10* (Wilson *et al.*, 1995), a basic domain at

the C-terminus implicated in the alignment of *p10* aggregates into fibrillar structures (Van Oers *et al.*, 1993) and a Proline rich domain that plays a role in the release of polyhedra from the infected nucleus (Zuidema *et al.*, 1993, Van Oers *et al.*, 1993).

Ecdysteroid UDP-glucosyltransferase (EGT) catalyses the glycolysation of insect moulting hormone, *i.e.* ecdysteroid (O'Reilly *et al.*, 1992). This inactivation of the hosts moulting hormones results in extended larval periods as moulting and pupation is either delayed or inhibited (O'Reilly, 1995), effectively prolonging the life-span of the insect (Clarke *et al.*, 1996). In final instar larvae, the expression of EGT can result in increased virus yield due to the lack of the feeding arrest normally associated with pupation (Clarke *et al.*, 1996). Hence, as mentioned elsewhere, the *egt* gene is normally the target for deletion to improve the insecticidal properties of the baculovirus. This deletion results in reduced feeding and earlier death of the infected insect (O'Reilly and Miller, 1991).

Programmed cell death or apoptosis appears to be an important cellular self-destruct mechanism that is activated in response to diverse signals during normal systemic and embryonic development, tissue homeostasis, cancer and disease pathogenesis (Douglas *et al* 1997; Birnbaum *et al* 1994; King *et al*, 1994). Apoptosis is one of the final host cellular defence mechanism against viral infection.

The inherent ability of a host cell to block viral infection by undergoing apoptosis and the antagonistic ability of the virus to block apoptosis, is indicative of the coevolution of virus and host. This virus-host interaction is crucial in the outcome of the virus infection at both the cellular and organismal level (Birnbaum *et al.,* 1994). Apoptotic inhibiting genes have been identified and characterised in

Adenoviridae, *Baculoviridae* and *Herpesviridae,* all of which function at either the signal transduction level or at the commitment stage of viral infection (Birnbaum *et al*, 1994). In *Baculoviridae* two different cellular apoptosis inhibiting genes have been reported, namely *p35* and *iap* (inhibitor of apoptosis). Each employs a different mechanism, indicating that each functions at conserved steps in the apoptotic pathway (Birnbaum *et al*, 1994).

The vast majority of vertebrate cells are capable of self-destruction by activation of an intrinsic cell-suicide programme when they have become obsolete or are damaged. This cell-suicide programme is commonly characterised by highly specific morphological and biochemical changes. In higher organisms apoptosis is usually associated with the initial activation of nucleases, which results in the condensation of both the nucleus and cytoplasm. Fragmentation follows and the dying cell is packaged into membrane-bound apoptotic vesicles, with subsequent phagocytosis and digestion by macrophages. It has been demonstrated in vertebrates that numerous intrinsic as well as extrinsic factors may act singly or in combination, as signal inducers of cellular apoptosis. Moreover, depending on the cell type, the same signal may either suppress or promote apoptosis (Steller, 1995). Baculoviruses are able to suppress apoptosis by the expression of anti-apoptotic genes (Du *et al.*, 1999), promoting viral replication and increased yield of virus progeny (Miller, 1997) Insect cells that are challenged by baculoviruses are signal-induced to undergo apoptosis (Lacount and Friesen, 1997). Currently, it is accepted that, similarly to vertebrate apoptosis, a combination of signals are required to induce baculovirus apoptosis in insect cells. Signal-inducers could include the activity of early transcriptional activators or DNA replication factors, virus-host cell receptor interactions, the process of viral DNA replication, virus-mediated shutoff of host RNA synthesis and the metabolic perturbations caused by

viral biosynthesis (Lacount and Friesen 1997).

Baculovirus induced apoptosis results in the complete cessation of all protein synthesis, a 100- to 1000-fold decrease in the budded virus production, and the absence of occlusion bodies (Birnbaum *et al* 1994). The baculovirus induced cell line- and host-specific apoptosis is characterised by the degradation of intracellular DNA into oligonucleosome-sized fragments, chromatin condensation, plasma membrane blebbing and premature cytolysis (Lacount *et al*, 1997).

The *p35* gene is transcribed in the early phase of gene expression (Hershberger *et. al.*, 1992), which precedes viral DNA replication (Miller, 1988). It is a trans-dominant factor that facilitates AcMNPV growth in a cell line-specific manner. The induction of apoptosis by *p35* mutants, exhibits cell line, tissue and species specificity (Prikhod'ko *et al*, 1996). Since the 35K protein has been implicated in apoptotic inhibition in phylogenetically diverse animals, such as nematodes and mammals, it has been proposed that it interacts with a component in the cell-death mechanism that has been phylogenetically conserved (Steller, 1995).

Inhibitors of Apoptosis (*iap*) are a class of genes found in both insect viruses and vertebrates (Dai *et al.*, 1999). All baculovirus IAP's identified to date share two structural features (Duckett *et al.*, 1996). They encode a RING finger motif (C3HC4) and two unique N-terminal Cys/His motifs known as Baculovirus IAP Repeats (BIRs) (Birnbaum *et al.*, 1994). It is believed that IAPs function early in the apoptotic pathway, probably inhibiting CED-3/ICE death protease together with other caspases (Dai *et al.*, 1999; Duckett *et al.*, 1996). The molecular mechanism of IAP is not completely understood, but the

discovery of cellular homologs with anti-apoptotic activity suggests that iap gene products are normal components of a host cellular apoptotic pathway that have been acquired and modified, by baculoviruses, to block cellular apoptosis during infection (Prikhod'ko *et al*, 1996).

1.12 Phylogeny

The evolution of baculovirus genes is constrained by their function. However, location of these genes on the genomes is not and could be an informative way of determining baculovirus evolutionary relationship (Chen *et al*., 1999). Since genomic organisation and gene phylogeny reflect a similar evolutionary history, both could be used for determining baculovirus phylogeny (Hu *et al*., 1998).

Baculovirus phylogenetic relationships have been proposed using the DNA and protein sequences from polyhedrin (Zanotto *et al*., 1993; Cowan *et al*., 1994), *egt* (Hu *et al*., 1997; Barret *et al*., 1995), DNA polymerase (Bulach *et al*., 1999), *gp37* (Jin *et al*., 1999), *gp41* (Liu and Maruniak, 1999) and *lef2* (Chen *et al*., 1999), to name but a few. Based on analysis of these sequences, NPVs have been divided into GroupI and GroupII (Zanotto *et al*., 1993). An understanding of these baculovirus phylogenetic relationships could be important in the future development of biopesticides and eukaryotic expression systems based on intertypic recombinants (Zanotto *et al*., 1993).

1.13 Research Rationale

In South Africa there are more than 106 insect pests that attack a wide variety of crops. The top ten or twenty of these can seriously limit successful production on the farm. Costs involved in controlling these pests are considerable, often higher than the value of the crop itself. *Trichoplusia ni* (common

name: cabbage looper) is a pest that can cause considerable damage to a wide variety of economically important crops. Although *Trichoplusia ni* has successfully been controlled with synthetic chemical pesticides, awareness about the negative impact of these control measures on the environment has necessitated the development of safer alternatives. Additionally, cabbage looper resistance to the commonly used pesticides has been reported.

Since a *Trichoplusia ni* multiple nucleopolyhedrovirus has previously been used in the effective control of the pest, the potential of characterizing a South African baculovirus isolate showed great potential. A latent baculovirus infecting a field population of *Trichoplusia ni* was isolated and characterised. Initial DNA and protein characterisation identified it as a novel baculovirus. The aim of this research was to characterise the baculovirus at a molecular level. This could lead to future improvement of the viral insecticidal properties.

The family Baculoviridae include more than 600 viruses with only nineteen receiving species status (Murphy *et al.*, 1995). The genome sequencing and mapping of NPVs could prove important in determining the relationships between these viruses. Additionally, it could be useful in understanding the importance of gene arrangement and the essential domains of genes. This could provide insight about the *cis*- and *trans*-regulation among genes (Jin *et al.*, 1997). **Chapter 2** deals with the determination of gene order and arrangement of the novel baculovirus isolated from a field population of *Trichoplusia ni*. Gene order could also be used as a phylogenetic marker in characterizing the relationship of TnSNPV to these other viruses.

Polyhedrin is the most conserved baculovirus protein sequenced and characterised thus far. It has been shown that polyhedrin is essential for the environmental persistence of the virus. Among lepidopteran baculovirus polyhedrin genes, amino acid identities are commonly in excess of 80%. Since the gene is not essential for infectivity or efficacy, foreign genes are often introduced *in lieu* of the coding region. Polyhedrin gene sequences have also been used to predict the phylogenetic relationship of baculoviruses (Zanotto *et al.*, 1993). The TnSNPV polyhedrin gene was sequenced as a prelude to the introduction of a foreign insect-specific toxin gene to increase the efficacy of the virus. Secondly, the gene sequence could be used to predict the putative phylogenetic classification of the virus (**Chapter 3**).

Polypeptide p10 is non-structural protein that is expressed late in the infection cycle. Various regions within the gene have been shown to confer unique functions on the virus (Wilson *et al.*, 1995). Similarly to polyhedrin, the peptide is not essential for viral infectivity. Hence, the strong promoter is often used for expression of eukaryotic proteins in expression vector systems. Although p10 has been conserved throughout baculovirus evolution, the primary peptide structure has not been conserved. This suggests that p10 evolved from an ancestor gene more rapidly than other baculovirus genes. The identification and characterisation of the TnSNPV p10 gene is described in **Chapter 4**. The gene was characterised as a prelude to using it in an eukaryotic expression vector system.

Comparative sequence analysis of specific regions of different baculoviruses has shown major differences in both gene content and arrangement (Theilmann and Stewart, 1992). Although the relationships between gene arrangement and content of MNPV genomes have been studied extensively, much less is known about the relationships between SNPVs and MNPVs. Such comparisons could

elucidate important information about baculovirus phylogeny. In **Chapter 5** a 3.0kb subclone of the genome was sequenced and analysed. Analysis of such a large fragment could also reveal why the genome of TnSNPV is so much larger than that of the prototype virus AcMNPV. A comprehensive knowledge of baculovirus phylogeny could be used to resolve the nomenclature of these viruses. Additionally, it could be useful in the future development of biological pesticides and eukaryotic expression systems based on intertypic recombinants (Zanotto *et al.*, 1993). In **Chapter 5** various TnSNPV genes were used to determine the phylogenetic relationship between TnSNPV and other baculoviruses. As mentioned earlier, this knowledge could prove invaluable in future development of TnSNPV as either, an improved pesticide, or expression vector system.

Chemical pesticides have proven detrimental to both the environment and to human health. Therefore, safer, cheaper alternatives are constantly being sought. Baculoviruses have successfully been used to control various insect pests in different countries, particularly Brazil and China. A baculovirus isolated locally could prove ideal for the effective control of insect pests. However, before any attempt is made to genetically improve such a virus, a detailed study of the molecular dynamics involved in the virus need to be studied extensively. This thesis could present an important foundation for such future works.

Chapter 2

Research Results

The identification and characterisation of a baculovirus isolated from a field population of *Trichoplusia ni*

Chapter 2

2.1 Abstract

A baculovirus isolated from a field population of *Trichoplusia ni* was isolated and characterised. Electron microscopy identified the virus as a single capsid nucleopolyhedrovirus. Protein and DNA restriction profiles differed significantly from the prototype baculoviruses BmSNPV and AcMNPV. Subsequently, Southern hybridisation was used to map the genome organisation of the *Trichoplusia ni* single capsid nuclear polyhedrosis virus (TnSNPV). A partial physical map was constructed for the enzymes *Eco*RI, *Pst*I and *Sac*I. End-terminal sequencing of the junctions between cloned fragments was used to verify the physical map. The viral genome was estimated to be approximately 158 kilobase pairs (kbps). Twenty-five putative open reading frames (ORFs) homologous to those of other baculoviruses were identified. The order of these ORFs was located in the TnSNPV genome and their arrangement compared to that of AcMNPV, SeNPV and LdMNPV. TnSNPV gene homology and arrangement were most similar to that of SeMNPV, possibly indicating that TnSNPV is a Group II NPV.

2.2 Introduction

The family *Baculoviridae* consists of two genera, the nucleopolyhedrovirus (NPV) and the granulovirus (GV). Although the family include more than 600 viruses, only nineteen have received species status (Murphy *et al.*, 1995). *Baculoviridae* are insect specific pathogens that contain genomes that range in size from 80-220kbs (Kool *et al.*, 1995; King and Possee, 1994; Arif, 1986). Limited DNA sequence homology is apparent between different baculovirus isolates (Gombart *et al.*, 1989), with little known about the genetic relationships between members (Jin *et al.*, 1997).

In vivo NPV replication gives rise to two phenotypically distinct viruses, budded virus (BV) and polyhedra-derived virus (PDV) (Jin *et al.*, 1997; King and Possee, 1994). Whereas, BV is released into the haemolymph and is responsible for the systemic spread of infection (Bonning and Hammock, 1993), PDV is released upon rupture of the cuticle ensuring the horizontal transfer of virus from insect to insect (Rohrmann, 1992; Miller, 1988). Although BV and PDV are genotypically identical, they differ in gross morphology, structural protein composition, source of virion envelopes and cell specificity (Hong *et al.*, 1994; Braunagel and Summers, 1994).

AcMNPV with a genome size of 128kbs (Vlak and Smith, 1982), is capable of encoding for at least 150 proteins (Kamita *et al.* 1993; Kool *et al.*, 1995). Since transfection of viral DNA into viable susceptible cultured insect cells lead to the production of complete infective virions, it is evident that under these conditions no virion-associated structural proteins are required for virus replication (Rohrmann, 1992). To date, only one PDV associated protein (p74) is known to be essential for successful infection of insects (Kuzio *et al.*, 1989).

Current evidence suggests that gene expression is not influenced by exact gene location on the genome or their proximity to other specific genes (Rohrmann, 1992). As mentioned earlier, AcMNPV is the most studied baculovirus, with the complete genome sequenced and mapped (Ayres *et al.*, 1994). Although 50 of the more than 150 open reading frames identified have been characterised in detail (Ayres *et al.*, 1994), the molecular mechanism of the AcMNPV life cycle is still under investigation. Thus, the genome sequencing and mapping of other NPVs could be useful in understanding the importance of gene arrangement and the essential domains of genes. This could provide insight into the *cis*- and *trans*-regulation among genes (Jin *et al.*, 1997).

In countries where silkworms have been cultivated commercially for centuries, it was noted that larvae could develop polyhedrosis disease from exposure to a variety of environmental stress factors (Volkman, 1997). Only with the advent of DNA analysis technology, however, could external contamination as the source of the infective agent be ruled out. This showed that the activation of a latent virus was involved in the initiation of disease. A latent infection could be defined as an infection that does not produce noticeable signs of disease, but can be transmitted to another host (Kukan, 1999). Latency is thought to provide an additional mechanism for the vertical transmission of baculovirus from one generation to another (Hughes *et al.*, 1993).

In this study, a baculovirus isolated from an asymptomatic field population of *Trichoplusia ni* was characterised. Electron microscopy identified the virus as a single capsid nucleopolyhedrovirus. Restriction enzyme profiles of genomic DNA appeared distinct from those previously published (Hughes and Wood, 1981). The TnSNPV genome was estimated to be about 158kbps. Southern

hybridisations were used to produce a partial genomic map and end-terminal sequencing of the *Eco*RI and *Pst*I libraries was used to construct a partial functional genomic map. These partial genomic maps were compared to those of AcMNPV, SeNPV and LdMNPV. The arrangement of genes was compared using GeneParityPlot analysis (Hu *et al.*, 1998). The arrangement and homology of the TnSNPV genes were most similar to that of SeMNPV, possibly indicating that TnSNPV is a NPV Group II baculovirus.

2.3 Methods

2.3.1 Infection of insect larvae

The virus was isolated from a field population of asymptomatic *Trichoplusia ni* (*Hübner: Noctuidae*) artificially infected with *Thysanoplusia orichalcea* single capsid nuclear polyhedrosis virus. Early third instar *T. ni* larvae collected from a farm in the Eastern Cape, South Africa were reared on a semi-synthetic lepidoptera diet (Appendix). Late fourth or early fifth instar larvae were infected with virus using the droplet feed method (Hughes and Wood, 1981). Briefly, larvae were fed on small plugs ($4mm^2$) of diet, containing unknown concentrations of polyhedra. Plugs of infected diet were dispensed into multiwell plates and single larvae were placed on top of the diet. Larvae were maintained at 25^0C, with a natural day-night cycle of 24 hours, after which time those larvae that had consumed the entire diet plug were transferred to fresh uninfected diet in glass containers. These larvae were incubated at 25^0C until death.

2.3.2 Virus isolation

Larvae exhibiting typical baculovirus-infection symptoms were crushed, diluted in approximately 20ml

0.1% sodium dodecyl sulphate (SDS) and filtered through three layers of muslin cloth. The extract was centrifuged at 15K for 30 minutes to pellet the virus. Resultant pellets were then resuspended in distilled water (dH_2O), layered onto a 45-60% (w/w) discontinuous sucrose gradient, and centrifuged in SW28 swingout-bucket rotors in a Beckman L7 Ultra Centrifuge at 7K for 90 minutes at 1000 microns vacuum. The respective viral bands were harvested, diluted in dH_2O and pelleted at 15K for 30 minutes. Pellets were resuspended in dH_2O and layered onto a 30-60% (w/w) continuous sucrose gradient. Viral bands were harvested, pelleted, resuspended in approximately 500µl of dH_2O.

2.3.3 Gross morphology

Purified virus was fixed in 2% gluteraldehyde overnight. These treated pellets were washed and then postfixed in 1% osmium tetroxide for a further 2 hours, and rinsed in distilled water. The pellets were dehydrated stepwise by increasing the concentration of alcohol, beginning with 30%, working up to 100% (in 10% increments) and finally transferred to acetone. Pellets were then embedded in a 50:50 acetone:spur resin mixture overnight, placed in fresh resin for 1 hour, followed by impregnation in fresh resin for a further 5 hours under vacuum and finally embedded in fresh resin. Final pellets were sectioned using a glass knife and the sections stained with uranyl acetate, and viewed under an electron microscope (*Jeol* 200CX).

2.3.4 Preparation of TnSNPV DNA

Viral DNA was extracted as described previously (Davis and Wood, 1996). Briefly, purified viral capsules ($2x10^6$OB/ml) were alkali lysed by addition of 0.5M Na_2CO_3 (final concentration) and incubated at 37^0C for 30 minutes. The OB concentration was estimated using light microscopy counts

with a haemocytometer. SDS was added to a final concentration of 0.1%, and the mixture was incubated at 60^{0}C for 30 minutes (or until clear), followed by cooling on ice. Proteinase-K (500µg/ml) was added and the mixture was incubated at 37^{0}C for 1 hour. Subsequently, the mixture was extracted with an equal volume of TE saturated-phenol (pH 8). The remaining DNA in the phenol layer was re-extracted with an equal volume of TE saturated-phenol. These two aqueous layers were pooled and extracted once more with phenol, once with phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1). DNA was precipitated by the addition of 1/10th volume NaAc (pH 5.5) and 2,5 volumes of ice-cold ethanol.

2.3.5 Restriction endonuclease digestion and construction of genomic DNA libraries

1µg DNA was digested with 10U of the following restriction enzymes: *Pst*I, *Eco*RI and *Sac*I, according to the specifications of the manufacturers, Boehringer Mannheim. The restriction fragments were separated by electrophoresis in a 0.8% and 1.0% horizontal slab agarose gel in 1xTAE at 40V for approximately 20 hours. Gels were stained with ethidium bromide and visualised by UV transillumination at 312nm. In order to create partial genomic libraries, the resultant digested DNA fragments were cloned into compatible sites of pBlueScript (Sambrook *et al.*, 1987).

2.3.6 Construction of the TnSNPV physical genomic map

Digested genomic DNA was transferred to Hybond-N nitrocellulose membranes (*Amersham*, Life Sciences) by capillary Southern blot techniques (Sambrook *et al.*, 1987). These blots were probed using DIG Nick Translation-labelled (Boehringer Mannheim) library clones. Prehybridisations and hybridisations were performed under stringent conditions, at 68^0 C for 2 and 18 hours respectively

(Appendix). Detection of results was done using the DIG-DNA Labelling and Detection Kit according to the manufacturers' instructions (Boehringer Mannheim) and visualised after 18 hours. Additionally, restriction digests, using *Eco*RI, *Pst*I and *Sac*I, were used to map the larger library fragments. Finally, end-terminal sequenced ("sniff-sequencing") of the partial library clones was used to verify the junctions between clones.

2.3.7 DNA sequencing and analysis

The genomic libraries were "sniff-sequenced" using an automated sequencer with universal primers. Nucleotide sequences were compared with those in GenBank at the National Centre for Biotechnology by using the Advanced Blast Search Server (Altschul *et al.*, 1997).

2.3.8 Arrangement of genes in the genome

To enable direct comparisons of gene arrangement among LdMNPV, AcMNPV and SeMNPV, only gene homologues identified in all four genomes were selected. To allow a computational comparison, the TnSNPV gene homologues of LdMNPV, AcMNPV and SeMNPV were renumbered starting with polyhedrin as number 1 and renumbering the remaining ORFs according to their sequential position on the respective linear genomes from left to right. These numbers were used as input for GeneParityPlot analysis (Hu *et al.*, 1998). A straight line indicates a collinear gene arrangement between the test virus and AcMNPV.

2.4 Results and Discussion

Insect populations are characterized by natural cycles of expansion and ensuing collapse in numbers

(Campbell, 1981; Elkinton and Liebhold, 1990). Many of these have been reported to be due to the sporadic outbreak of either a latent NPV or cytoplasmic polyhedrosis virus (CPV) (Longworth and Cunningham, 1968; Evans and Harrap, 1982). Various environmental and stress factors have been thought to activate these latent viruses (Mckinley *et al.*, 1981; Hughes *et al.*, 1993), but only with the advent of DNA analysis technology, could latency be demonstrated.

Latency requires the pathogen to be in a noninfective, nonprogenetive state in the host without causing disease. Under various stress factors, the pathogen is transformed to an infective, reproductive state (Fuxa *et al.*, 1992). Kukan (1999) suggested that baculoviruses remained latent by either integrating the viral DNA into the host genome as found in hepatitis B infection (Howard, 1986), or that the virus could be maintained as independent viral genetic material in the host cell nucleus as with herpes simplex (Mellerik and Fraser, 1987). There is some, although speculative evidence that these latent viruses exist as persistent infections (Hughes *et al.*, 1997), by remaining at low levels of infection with the expression of viral genes. However, as yet, the exact mechanism of baculovirus latency is not understood.

In the present study the activation of a latent *Trichoplusia ni* virus is reported. The virus could have been activated by feeding the fifth instar field collected *Trichoplusia ni* larvae a heterologous NPV (Hughes *et al.*, 1993; McKinley *et al.*, 1981; Ponsen and de Jong, 1964; Kelly *et al.*, 1981; Smith, 1963), in this instance a *Thysanoplusia orichalcea* NPV (ToSNPV). Equally, the virus could have been activated by exposure to various stress factors such as extreme temperature changes, overcrowding, changes in humidity, changes in day-night cycle or a change in diet (Volkman, 1997). The *Trichoplusia*

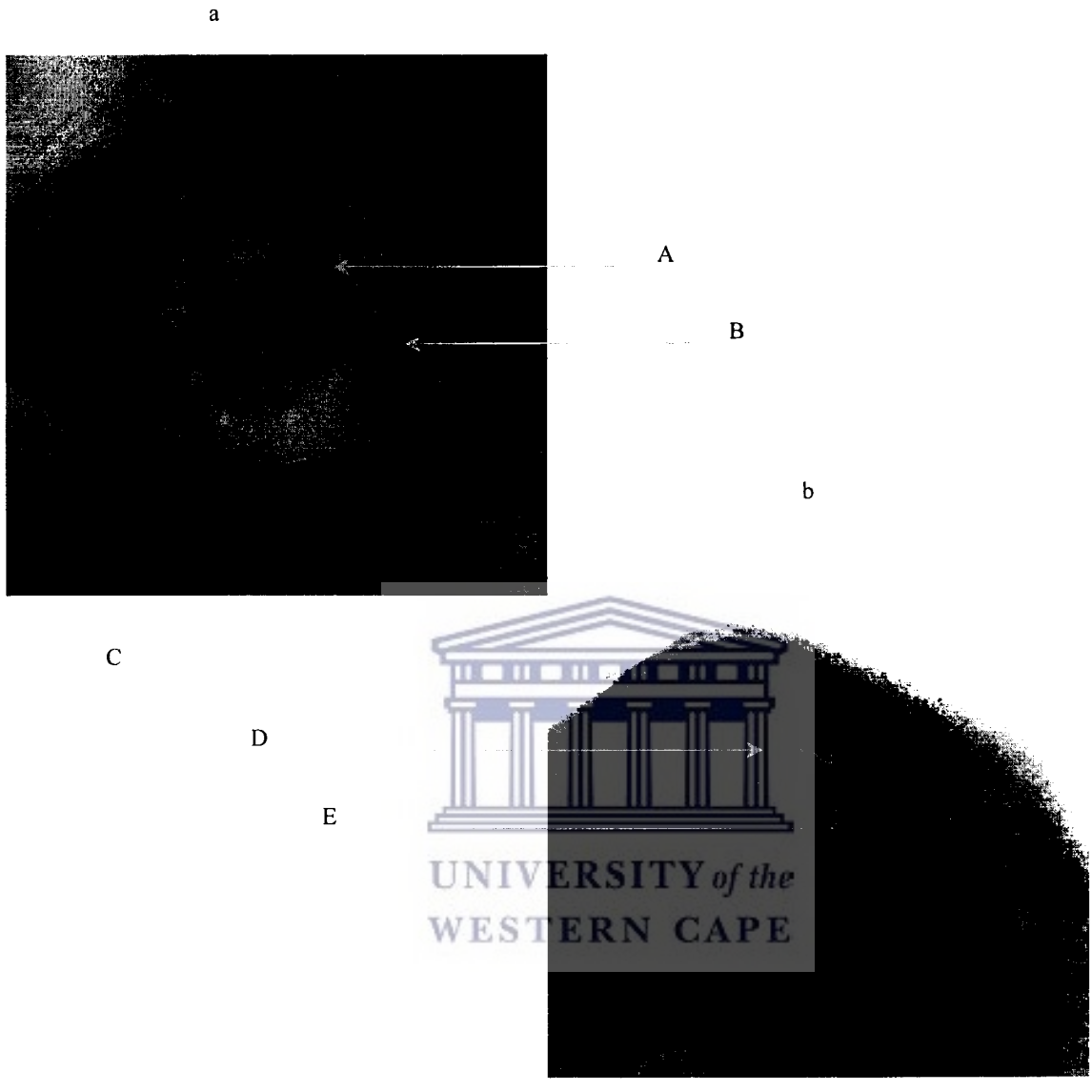


Figure 2.1: a) Transmission electron micrograph (TEM) of TnSNPV. Virions (A) are embedded in a protein crystalline matrix (B), composed primarily of polyhedron (3 300x magnification). b) TEM of a section of TnSNPV. Single virions (D) enveloped in a proteinaceous membrane (E) surrounded by a polyhedrin envelope (C), are embedded in a protein crystalline matrix, composed primarily of polyhedron (135 000x magnification).

ni virus was shown to contain DNA endonuclease restriction and protein profiles distinct from that of ToSNPV (results not shown) and the previously characterised TnSNPV (Davis and Wood, 1996). Both ToSNPV and TnSNPV contained genomes approximately 40kbps smaller than the newly isolated virus. (Figure 2.2). Although GVs consisting of multiple enveloped nucleocapsids per occlusion body have been reported (Arnott and Smith 1968; Smith *et al.*, 1990; Crook and Brown, 1982), they normally consist of a single occluded virion (Kang *et al.*, 1997). The enveloped nucleocapsid with average dimensions of 30-60nm by 260-360nm, is embedded within an ellipsoidal protein matrix composed primarily of granulin. The occluded virion forms a structure approximately 300 to 500nm in length (Rohrmann, 1992) by 120 to 350nm in width (Tweeten *et al.*, 1981). On the other hand, NPVs polyhedrin crystallises around enveloped nucleocapsids, either as single (SNPV) or multiple (MNPV) nucleocapsids within the envelope. These occlusion bodies of PDVs are typically 1 to 15μm in diameter (Rohrmann, 1992), with the predominant size range between 0.6μm to 2.5μm. Functionally, these occlusion bodies are similar to those of two other insect viruses, the *Reoviridae* and the *Poxviridae* (Rohrmann, 1992). The rod-shaped nucleocapsids with dimensions of approximately 40nm - 60nm by 250nm - 300nm, may range in number from 1 to 29 per virion (Adams and McClintock, 1991). The electron microscopy study carried out here has identified the *Trichoplusia ni* virus as a single nucleocapsid nuclear polyhedrosis virus (TnSNPV) (Figure 2.1). The TnSNPV polyhedra with average diameters of about 16μm, contained nucleocapsids with dimensions of approximately 30nm by 200nm. Also, the enveloped nucleocapsid averaged about 300nm in length.

Restriction endonuclease (REN) digestion with *Eco*RI, *Pst*I and *Sac*I produced 27, 29 and 26 fragments, respectively. Restriction profiles of the TnSNPV genomic DNA are shown in Figure 2.3 and the sizes

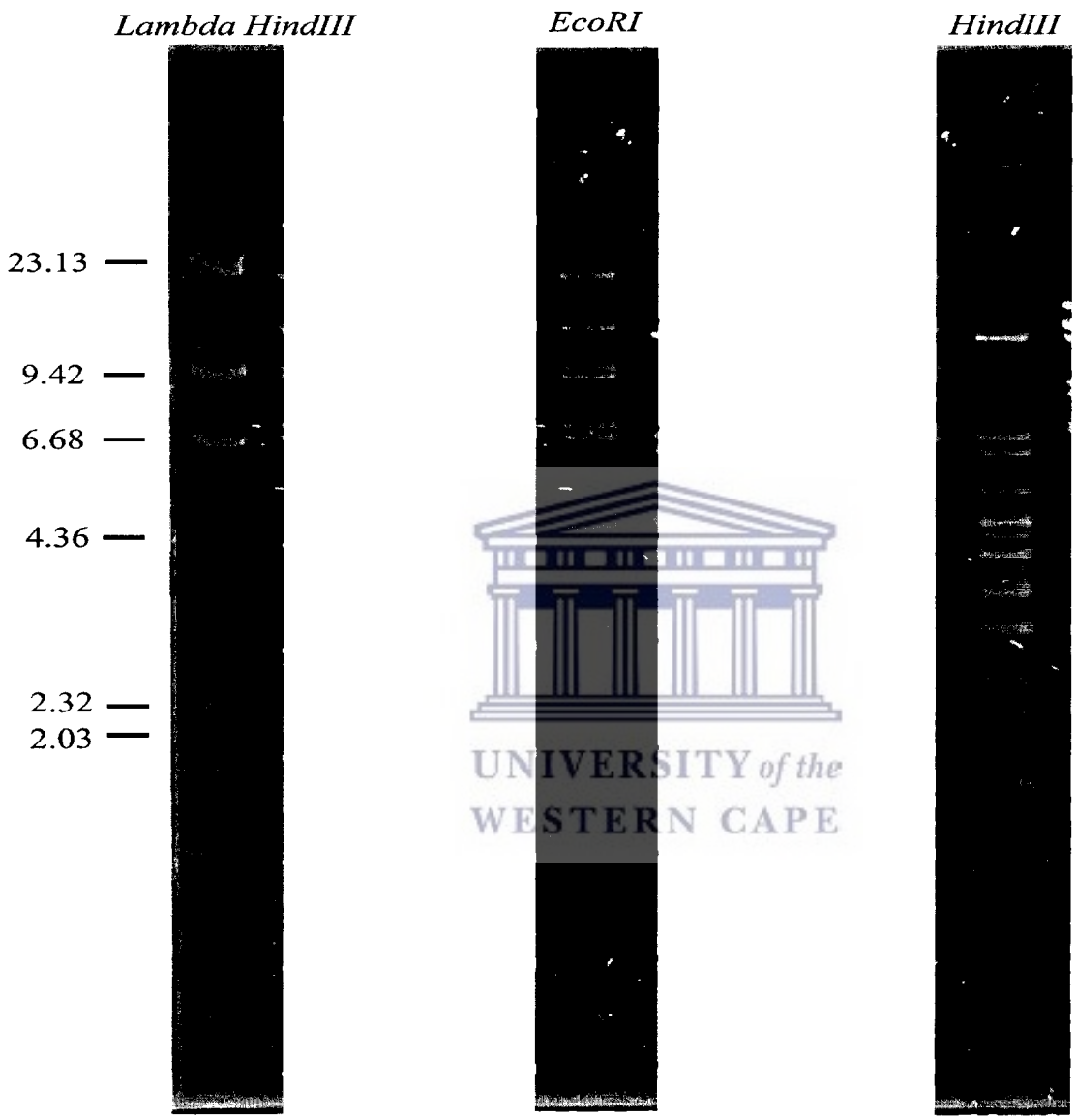


Figure 2.2: ToSNPV genomic DNA digested with *Eco*RI and *Hind*III. Lambda DNA digested with *Hind*III was used as the molecular marker (the sizes of the fragments are included).

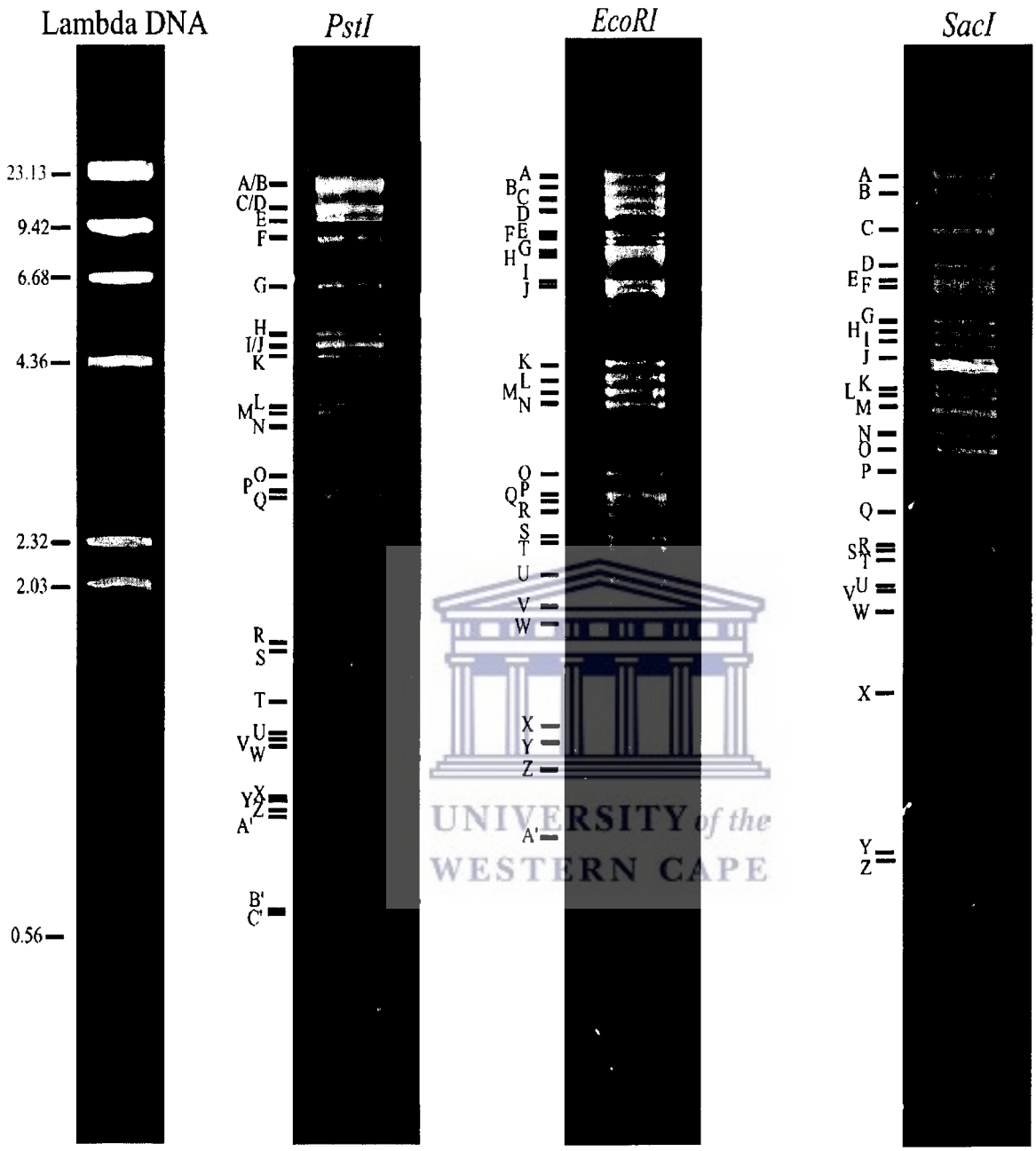


Figure 2.3: TnSNPV genomic DNA digested with *Eco*RI, *Pst*I and *Sac*I. Fragments are named alphabetically, starting with A for the largest. Lambda DNA digested with *Hind*III was used as the molecular marker (the sizes of the fragments are included).

Table 2.1: Size of restriction endonuclease fragments in kb of TnSNPV genomic DNA

Fragment	Enzyme		
	PstI	EcoRI	SacI
A	22.47	22.47	34.68
B	22.47	16.54	20.09
C	15.20	14.04	12.17
D	15.20	12.17	8.62
E	13.04	**10.11**	7.84
F	10.72	9.56	7.50
G	7.18	8.62	6.89
H	5.51	8.21	5.91
I	5.15	6.61	5.51
J	5.15	**6.36**	5.15
K	**4.44**	4.30	4.69
L	3.85	4.06	4.18
M	3.76	3.85	3.96
N	3.49	3.57	3.76
O	2.90	2.78	3.40
P	2.78	2.56	3.25
Q	2.72	2.51	2.97
R	1.76	2.46	2.56
S	1.73	2.28	2.33
T	1.50	2.20	2.20
U	1.39	1.98	2.05
V	1.35	1.88	1.88
W	1.31	1.78	1.76
X	1.19	**1.35**	1.52
Y	1.14	1.31	1.03
Z	1.12	1.24	1.02
A'	1.03	0.99	
B'	0.97		
C'	0.90		
Total:	156.92	157.70	157.91

Values in bold indicate clones that have been completely sequenced.

Values underlined have been "sniffed-sequenced".

of the fragments are summarised in Table 2.1. Fragment sizes were calculated from 0.8% and 1.0% agarose gels using the DNAFRAG v3.03 program (Schaffer and Sederoff, 1981) and sizes were adjusted according to the sequencing data obtained. TnSNPV contains a genome of approximately 158kbps, which is significantly larger (±30kbps) than the genomes of AcMNPV, OpMNPV, SeMNPV and BmNPV (Ayres *et al.*, 1994; Ahrens *et al.*, 1997; Ijkel *et al.*, 1999; Maeda, GenBank L33180). This is, however, similar in size to the genome of LdMNPV (Kuzio *et al.*, 1999). Ten of the 29 *Pst*I and 22 of the 27 *Eco*RI fragments were cloned into pBluescript (+) and "sniff-sequenced". These clones covered 17% and 71% of the viral genome, respectively. No obvious submolar bands were observed in the REN profiles (Figure 2.3). However, since the baculovirus has been isolated from field-collected larvae, the homogeneity of the virus sample needs to be tested. This could be achieved by *in vivo* cloning procedures (Smith and Crook, 1988) or *in vitro* plaque purification techniques (King and Possee, 1992).

Using the partial genomic libraries and "sniff sequencing" data, a partial physical map was constructed for *Bam*HI, *Sac*I and *Eco*RI (Figure 2.4). Since *Pst*I fragments A and B were equimolar, they are represented by (A/B)' and (A/B)''. Similarly, *Pst*I fragments C and D are represented by (C/D)' and (C/D)", as well as I and J by (I/J). Where possible, "sniff sequencing" data of separate clones were used to reconstitute possible ORFs identified by homology searches. This was used to verify certain sections of the map generated by Southern hybridisations. The one end of *Pst*I fragment (A/B)', containing the entire polyhedrin gene, was chosen as the start of the circular map (Vlak and Smith, 1982). This positioned *p10* at approximately 70 map units (m.u.) to the right of polyhedrin. Large portions of the map have to still be resolved. The order and orientation of the majority of fragments needs also to be

confirmed. Only 76% of the *Pst*I map has been completed, with even lower values for the *Eco*RI (56%) and *Sac*I (57%) maps. Even so, this partial map can be used to elucidate important information about the genetic make-up and evolutionary relationship of TnSNPV, a novel virus.

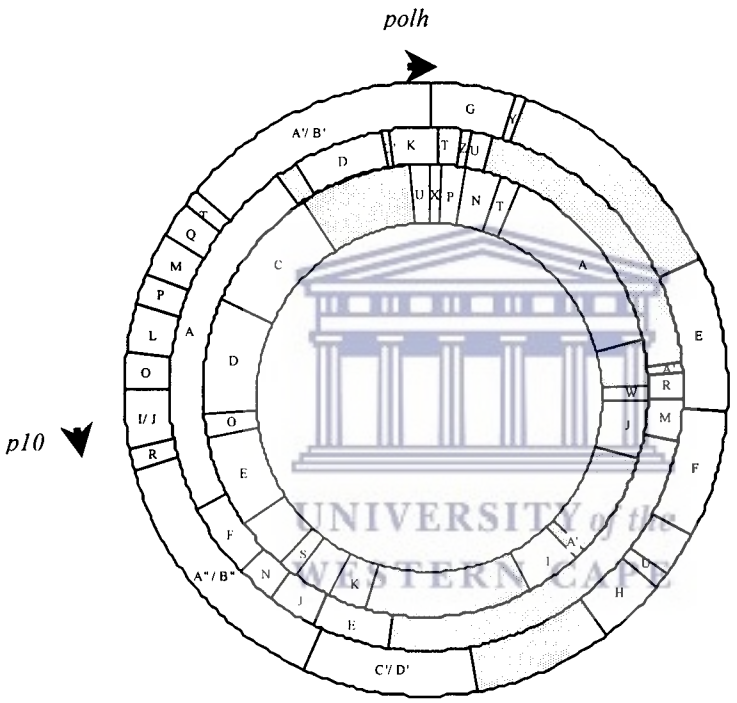


Figure 2.4: Circular partial physical map of the TnSNPV genome with restriction endonuclease sites for *Eco*RI (inner), *Pst*I (outer) and *Sac*I (middle). Restriction sites are indicated in kb from the zero point. The location and the direction of the polyhedrin and *p10* genes are shown. Shaded areas indicate gaps.

Although about seven hundred and forty four ORFs have been identified in the five MNPV genomes sequenced to date, the genomes have a cumulative total of only 245 different ORFs. Eighty five of these ORFs are conserved amongst the five genomes (Ayres *et al.*, 1994; Ahrens *et al.*, 1997; Kuzi *et al.*, 1999; Gomi *et al.*, 1999; Ijkel *et al.*, 1999), with possible functions assigned to half of these. These conserved ORFs could be considered the core baculovirus genes (Ijkel *et al.*, 1999), required for basic virus features such as structure, transcription, efficacy, replication and auxiliary functions. Also, a number of unique ORFs have been identified in each of the genomes. An estimated 30 kbps of the TnSNPV genome has been sequenced, mostly as single stranded DNA. This represents about 19% of the estimated 158kbp genome (Table 2.1). Analysis of the sequence data identified 25 ORFs homologous to baculovirus sequences (Table 2.2), seven of which have been sequenced as double stranded DNA. These ORFs included the following conserved baculovirus genes: polyhedrin (*polh*), late expression factor 6 (*lef*-6), *p26*, fibrillin (*p10*), *p74*, immediate early gene 1 (*ie*-1) and an SeORF-128 homologue. All the putative TnSNPV ORFs, with the exception of SeORF-128, contained either the late or early transcription initiation motifs (TAAG or CAGT and TATA, respectively) upstream of the translation start codons. With the exception of polyhedrin, the seven putative TnSNPV ORFs consistently showed greater identity values to homologues from SeMNPV (Figure 2.5). This could indicate a possible evolutionary relationship between SeMNPV and TnSNPV. As yet, no unique ORFs have been identified in the TnSNPV genome. Auxiliary genes are not essential for replication, but confer selective advantages on the virus in nature (O'Reilly, 1997). The auxiliary genes *sod*, *ptp* and ecdysteroid UDP-glucosyltransferase (*egt*) have been identified on the genome of TnSNPV (Table 2.2). *Egt* was amplified by polymerase chain reaction and the amplicon was used as a probe to identify the location of the putative ORF on the partial genomic map.

Table 2.2: Identified putative ORFs in the TnSNPV genome and their homologues in AcMNPV, SeNPV and LdMNPV (not shown in any particular order)

Ac ORF	**Se ORF**	**Ld ORF**	**Name ORF**	**Abbreviation**
8	1	1	polyhedrin	*ph*
50	112	51	late expression factor 8	*lef*-8
103	62	104		*p48*
N/A	N/A	N/A	Op unknown protein	
139	7	23		*me-53*
147	132	15	major transactivator	*ie*-1
28	127	38	late expression factor 6	*lef-6*
138	131	27		*p74*
137	130	41	fibrillin	*p10*
136	87/129	40		*p26*
35	123	43	viral ubiquitin	*v-ubi*
3	N/A	N/A	Ac p17.0kd	
131	46	136	calyx protein	*pep*
31	48	145	superoxide dismutase	*sod*
95	70	97	DNA helicase	
1	N/A	N/A	protein tyrosine phosphatase	*ptp*
94	71	96	ODV protein	*odv-e25*
14	14	123	late expression factor 1	*lef*-1
29	128	39	Se-ORF128	
101	64	102	Se-ORF64	
146	133	16	Ld-ORF16	
N/A	45	120	ribonucleotide reductase small subunit	*rnr2*
133	41	157	alkaline exonuclease	*alk-exo*
53	108	54	LdORF-54	
107	N/A	55	LdORF-55	

Identity values between homologous baculovirus genes could be used to propose evolutionary relationships (Jin *et al.*, 1997) and could provide insight into possible gene function (Hu *et al.*, 1998). Seven of the TnSNPV ORFs sequenced were used to compare the homology of TnSNPV to AcMNPV, LdMNPV and SeMNPV (Figure 2.5). Different homology patterns were observed when the different baculovirus pairs were compared. Hu *et al* (1998) reported that pairwise comparisons could be used to

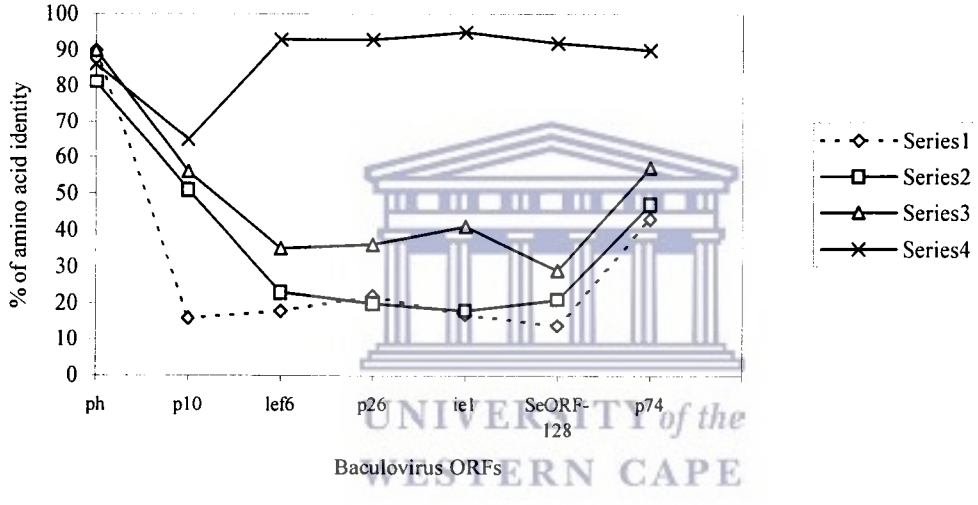


Figure 2.5: Pairwise comparison of gene homology of six ORFs of AcMPNV (Ac), BmNPV (Bm), TnSNPV (Tni), SeNPV (Se), and LdMNPV (Ld). *ph*: polyhedrin; *p10*: fibrillin; *ie*-1: immediate early gene 1; *lef-6*: late expression factor 6; *p74*; *p26* ; SeORF-128. Series 1: Tni/Ac; Series 2: Tni/Ld; Series 3: Tni/Se; Series 4: Bm/Ac

identify closely related baculoviruses when sequence data of poorly conserved genes were available. The extent of gene homology would differ more significantly between less related viruses, than between

more closely related viruses (Hu *et al.*, 1998). When the BmNPV and AcMNPV homologues were compared, high identity values were observed. Since both AcMNPV and BmNPV are Group I baculoviruses, Zanotto *et al.*, 1993; Cowan *et al.*, 1994), the higher homology values were to be expected (Hu *et al.*, 1998; Ijkel *et al.*, 1999). However, when comparing different Group II baculoviruses, lower identity values have been reported. This could be explained by the fact that NPV

Table 2.3 Sequential order of baculovirus homologues on the genomes of four NPVs used in GeneParityPlot analysis.

Gene Homologue	TnSNPV	LdMNPV	SeMNPV		AcMNPV
polh	1	1	1	1	1
egt	2	10	3	3	2
v-ubi	3	9	7	5	6
lef-6	4	5	6	6	3
SeORF-128	5	6	8	7	4
p26	6	7	5	8	7
p10	7	8	9	9	8
p74	8	4	10	10	9
ie-1	9	2	11	11	11
me-53	10	3	2	2	10
sod	11	11	4	4	5

*ORFs were renumbered in ascending order, starting with polyhedrin at #1 and then according to their sequential order from left to right on the genomes. Since SeMNPV contained 2 copies of p26, renumbering was repeated with the exclusion of one of the ORFs for each count.

Group II consists of three distinct subgroups (A, B and C) and a distantly related group of viruses (Bulach *et al.*, 1999; Chapter 5). Thus, the NPVs in Group I are more closely related than those in Group II. As mentioned earlier, the TnSNPV ORFs showed consistently higher identity values to

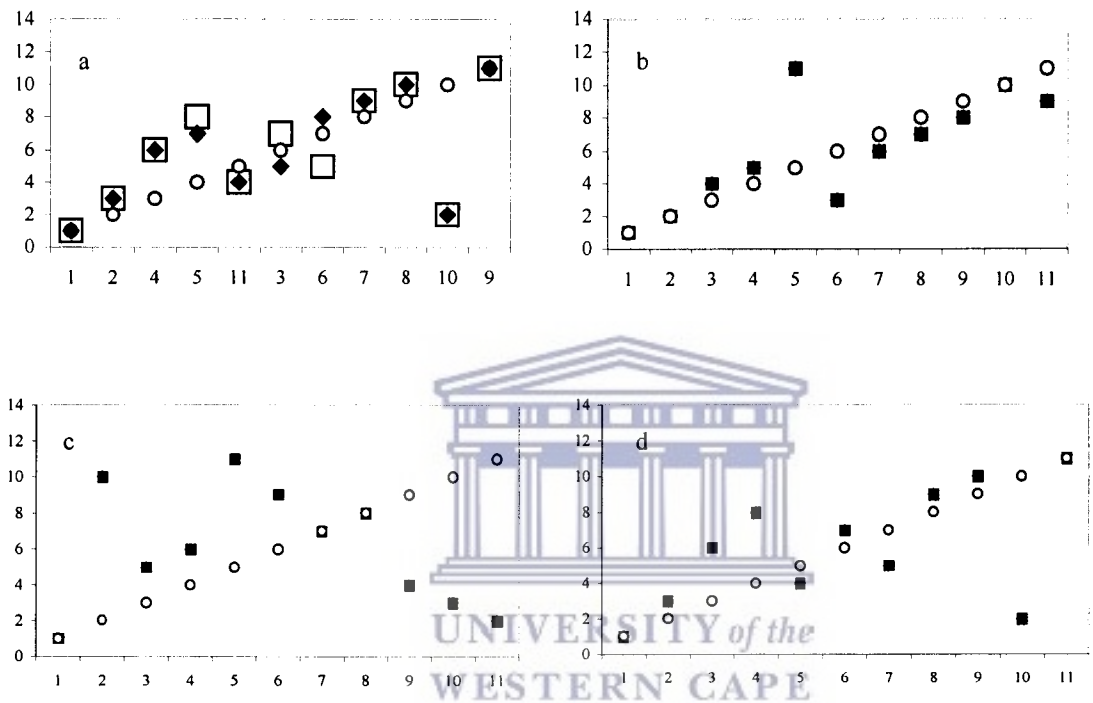


Figure 2.6: Graphic representation of the collinearity of baculovirus genomes obtained by GeneParityPlot analysis (Hu *et al.*, 1998). a) TnSNPV vs SeMNPV; b) TnSNPV vs AcMNPV; c) TnSNPV vs LdMNPV; d) SeMNPV vs AcMNPV. The straight line (o) represents the reference plot when two viruses have identical gene arrangement. The plot (b, c, d) represented by (■) indicates the collinearity between the two viruses compared. In (a) the (□) represents the plot including Se-*p26* (ORF-82) and (♦) represents the plot including Se-*p26* (ORF-129).

homologues in the genome of the Group II SeMNPV (Cowan *et al.*, 1994). Lower identity values were observed, however, when the TnSNPV ORFs were compared to homologues in the genome of LdMNPV, also a Group II baculovirus (Bulach *et al.*, 1999). Even lower identity values were noted when TnSNPV and AcMNPV homologues were compared. This provides tentative evidence that TnSNPV is a member of NPV Group IIA. The organization of genes in baculovirus genomes has been studied extensively (Jin et al., 1997). Finding conserved clusters of genes in baculoviral genomes could provide insight into the evolution of their genomes (Hu *et al.*, 1998). Additionally, gene clustering could be important for virus replication.

GeneParityPlot analysis has been used to determine such evolutionary relationships between baculoviruses (Hu *et al.*, 1998; Ijkel *et al.*, 1999). With the exception of a small number of insertions or deletions, the order of genes on the genomes of AcMNPV, BmNPV and OpMNPV is similar (Ahrens *et al.*, 1997; Ijkel *et al.*, 1999). Ten TnSNPV homologous to ORFs in AcMNPV, SeMNPV and LdMNPV were selected for comparison by GeneParityPlot analysis (Table 2.3). Not only has the exact position of these ORFs been determined on the TnSNPV genome, but also the ORFs are present in the genomes of all three selected MNPVs. The three MNPVs were chosen as representative members of NPV Group I, Group II and the distantly related Group II baculoviruses, respectively (Zanotto *et al.*, 1993; Bulach *et al.*, 1999).

TnSNPV has a rather different gene arrangement compared to LdMNPV, with the gene arrangement of TnSNPV and SeMNPV most similar (Figure 2.6). When SeMNPV ORF-129 (*p26*) was included in the comparison the similarity in gene arrangement between SeMNPV and TnSNPV was even higher. The

similarities between AcMNPV and TnSNPV gene arrangement were higher than expected. Hu *et al* (1998) reported lower similarities between gene arrangement of Group I and Group II baculoviruses. Thus, it appears as though the ORF sampling number used in this study was too small to make accurate comparisons between NPV from Group I and Group II. With the exception of LdMNPV, the clustering of *p26-p10-p74* has been conserved in most baculoviruses. However, the relationship between these genes must still be determined.

Gene arrangement and homology has been used as phylogenetic markers (Hu *et al*., 1998; Ijkel *et al*., 1999). In this study, analysis of arrangement and homology has been used to classify TnSNPV as a putative Group II NPV. Before any subsequent analysis or modification of the TnSNPV genome is to be attempted, the purity of TnSNPV has to be determined using *in vitro* techniques. However, initial attempts to introduce and propagate the virus in TN-365 (cells supplied by Boyce-Thompson Institute) were unsuccessful. Thus, the search for a susceptible cell line should be of primary concern.

Chapter 3

Research Results

The characterisation and phylogenetic relationship of the *Trichoplusia ni* single capsid nucleopolyhedrovirus polyhedrin gene

A portion of this chapter has been published as:

Fielding B.C. and Davison S. 1999. The characterisation and phylogenetic relationship of the *Trichoplusia ni* single capsid nucleopolyhedrovirus polyhedrin gene. *Virus Genes* 19, 67-73.

The nucleotide data reported in this chapter have been submitted to GenBank and has been assigned the accession number AF093405.

Chapter 3

3.1 Abstract

The polyhedrin gene was identified from the *Trichoplusia ni* (Tni) single capsid nucleopolyhedrovirus (SNPV). An *Eco*RI fragment containing the truncated polyhedrin gene was detected by hybridisation with an AcMNPV expression vector probe; the remaining portion was amplified by inverse PCR. An open reading frame (ORF) of 741 nucleotides (nt), encoding a putative protein of 246 amino acids (a.a) with M_r 28,780 Da was identified. The 5'-noncoding region contained the putative late (TAAG) transcription initiation motif. The 3' end, downstream of the translation stop codon, lacked an obvious putative poly (A) signal. Nucleotide and amino acid homology are greater than 90% to that of *Thysanoplusia oricalchea* MNPV polyhedrin sequences. Results suggest that TnSNPV is a member of the Group II nucleopolyhedroviruses.

3.2 Introduction

The Family *Baculoviridae* are invertebrate-specific pathogens that contain a single circular supercoiled double stranded DNA molecule that ranges in size from 80-220 kilobases (Miller, 1988). In excess of 600 insect species are susceptible to baculovirus infection (Rohrmann, 1992), with a single virus isolate normally restricted to one host or a few closely related species (Maeda, 1995).

The nucleopolyhedrovirus (NPV) genome is complex and is capable of encoding for at least 150 proteins (Kamita *et al.*, 1993; Maeda, 1995; Federici and Maddox, 1996), of which polyhedrin is hyperexpressed very late in the infection cycle (Vlak *et al.*, 1988). Polyhedrin, a protein approximately 29-kD in size (Rohrmann, 1992), is the primary constituent of the crystalline matrix surrounding enveloped nucleocapsids or occluded viruses (Gombart *et al.*, 1989; Weyer and Possee, 1989). Since non-occluded viruses have been found to be rapidly inactivated in the soil and on plant tissues as well as in dead larvae (Blissard and Rohrman, 1990), it is accepted that polyhedrin is essential for the persistence of the biological activity of the virus in the environment (Thiem, 1997).

Homology studies have identified polyhedrin as the most conserved baculovirus protein so far characterised. Among lepidopteran baculovirus polyhedrin genes, amino acid identities are consistently in excess of 80% (Rohrmann, 1992). Additionally, in excess of 50% identity has been reported between lepidopteran granulovirus and NPV occlusion protein sequences (Rohrmann, 1986). The introduction of foreign genes *in lieu* of the polyhedrin gene to increase the efficacy of the virus has resulted in varying success (Maeda, 1995). A biologically active mite neurotoxin expressed by a genetically modified AcMNPV, induced insect paralysis at a faster rate than the wild type virus (Tomalski and

Miller, 1991). Similarly, the use of a recombinant virus, producing an active scorpion toxin, reduced crop damage caused by the pest (Cory *et al.*, 1994; Steward *et al.*, 1991). However, AcMNPVs expressing active *Bt*-toxins were reported to be no more virulent than the wild-type virus (Merryweather, 1990).

Based on phylogenetic studies using sequences from polyhedrin (Cowan *et al.*, 1994; Zanotto *et al.*, 1993) and ecdysteroid glucosyl transferase (Clarke *et al.*, 1996; Barrett *et al.*, 1995) genes, NPVs have been classified into two groups, namely Group I and Group II. This suggests NPV evolution comprises of two distinct branches (Cowan *et al.*, 1994; Zanotto *et al.*, 1993). It appears as though the different lepidopteran NPVs evolved from a common lepidopteran NPV ancestor, rather than from cross-infection from arthropods (Rohrmann, 1992; Cowan *et al.*, 1994). However, conclusive support for this hypothesis can only be gained through continued molecular analysis of various other conserved baculovirus genes.

In this study, the polyhedrin gene of a nucleopolyhedrovirus isolated from a field population of *Trichoplusia ni* was characterised as prelude to the introduction of a foreign gene to increase the efficacy of the virus. The *Eco*RI-X fragment of 1.35kbs, shown to hybridise to a portion of the AcMNPV polyhedrin gene, was cloned and sequenced. The predicted polyhedrin gene product is compared to other occlusion proteins and a phylogenetic relationship is proposed. The nucleotide and amino acid sequences were most homologous (92% and 97%, respectively) to that of *Thysanoplusia oricalchea* MNPV. Sequence comparisons suggest that TnSNPV is a member of the previously defined Group II nucleopolyhedroviruses.

3.3 Methods

3.3.1 Viruses and insect hosts

Virus was isolated from *Trichoplusia ni* (*Noctuidae: Lepidoptera*) larvae collected in the Eastern Cape, South Africa. Propagation of virus was done using second instar larvae reared at 26^{0}C (artificial 12 hour day-night cycle; 65% humidity) on an artificial lepidopteran diet (Appendix). Virus killed larvae were collected and virus capsules isolated and purified as previously described (Crook, personal communication).

3.3.2 DNA extraction and digestion

Purified viral capsules were alkali lysed (Crook and Brown, 1982) and phenol extracted. Extracted DNA was digested with *Eco*RI, *Pst*II, *Dra*I and *Xba*I according to the manufacturers' instructions (Boehringer Mannheim) for 1.5 hours at 37^0 C.

3.3.3 Clone construction and Location of polyhedrin gene

*Eco*RI restriction fragments were cloned into the compatible site of the multiple cloning region of SK$^+$-BlueScript and transformed into supercompetent *E. coli* JM 105 cells (Sambrook *et al.*, 1987). Following blue-white selection, plasmid DNA was extracted by the alkaline lysis (Sambrook *et al.*, 1987). Positive clones were *Eco*RI-digested, run on 0.8% agarose gels, visualized and transferred to Hybond-N nitrocellulose membranes (*Amersham*, Life Sciences) by capillary Southern Blot techniques; these were routinely screened for the presence of the polyhedrin gene by hybridisation to a labelled probe. The probe consisted of an expression vector (pAcRP23), containing a portion of the AcMNPV polyhedrin gene, labelled using the DIG Nick Translation Mix kit (Boehringer Mannheim) according to

the manufacturers' specifications. Prehybridisations and hybridisations were performed under stringent conditions, at 68^{0}C for 2 and 18 hours respectively. Detection of results was done using the DIG-DNA Labelling and Detection Kit according to the manufacturers' instructions (Boehringer Mannheim) and visualised after 18 hours.

3.3.4 Sequencing and homology searches

Sequencing of the *EcoRI-X* fragment was done by automated sequencing using a Pharmacia ALF/Express automated sequencer, with universal Forward and Reverse primers (Figure 3.1). Amino acid and nucleotide sequences were compared with those in GenBank at the National Centre for Biotechnology by using the Advanced Blast Search Server (Altschul *et al.*, 1997). Initial results indicated an incomplete polyhedrin ORF at the 3' end of the sequence. PCR primers were designed, (Forward 5'-CCAATTGTACGCGGGCTGTTCTG-3'; Reverse 5'-CCGATTATGTTCCTCACGAGGTG-3') and the missing sequence amplified by inverse PCR methods (Sambrook *et al.*, 1987), purified, polished and cloned for sequencing (Stratagene).

PCR conditions:

Initial Denaturation	95^{0}C	1min	1 cycle
Denaturation	94^{0}C	1min	30 cycles
Annealing	60^{0}C	30sec	30 cycles
Primer Extension	72^{0}C	1min	30 cycles
Final Primer Extension	72^{0}C	5min	1 cycle

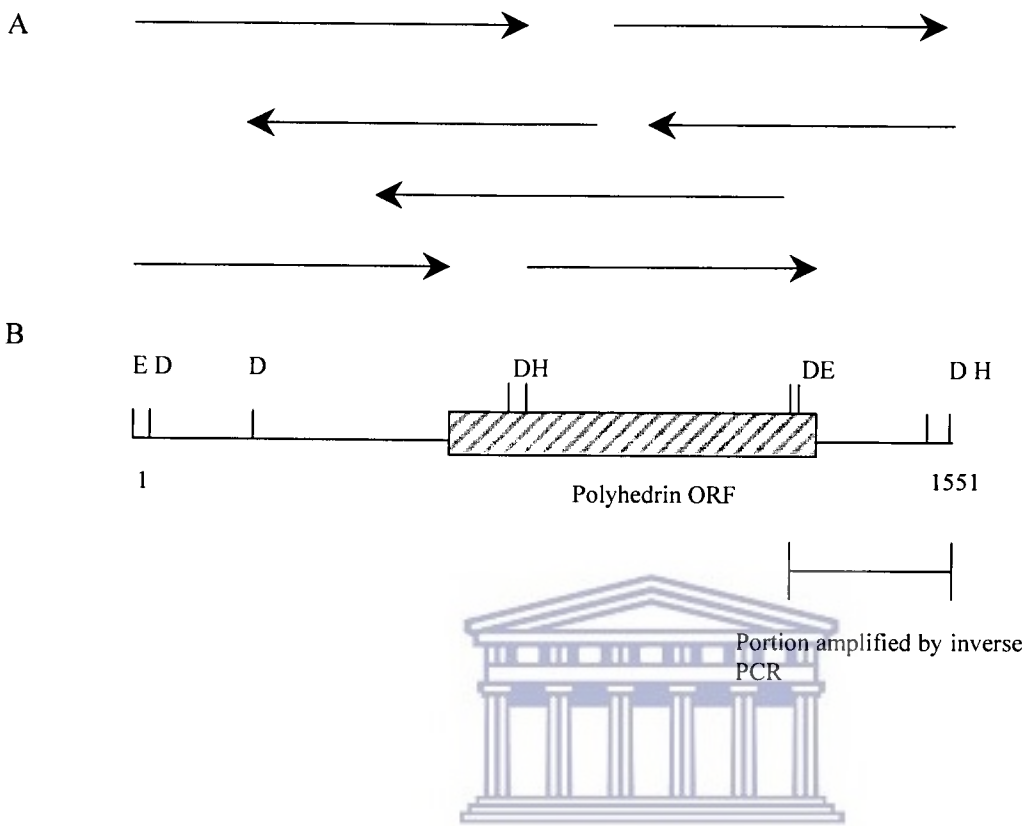


Figure 3.1: Enzyme map, sequencing strategy and ORF distribution of the TnSNPV DNA region. (A) Sequencing strategy. Deletion clones are represented by arrows starting at the beginning of the insert and pointing in the sequencing direction. (B) Map of selected restriction enzyme recognition sites; E = *Eco*RI; H = *Hind*III; D = *Dra*I. (Not drawn to scale)

3.3.5 Purification of the amplicon by selective precipitation

One volume ammonium acetate (4M) was added to the PCR products in a microcentrifuge tube, followed by the addition of 2,5 volumes of 100% ethanol (v/v) at room temperature (RT) and centrifuged immediately at 10 000xg for 20 minutes at RT. The supernatant was removed and the resultant DNA pellet was washed with 200 µl 80% (v/v) ethanol, centrifuged 10 000xg for 10 minutes

at RT and the supernatant removed. The pellet was dried for 10 minutes at 37^{0}C and resuspended to the original volume using TE buffer.

3.3.6. Creating flush ended PCR products

The following components were added (in order) to a microcentrifuge tube. Firstly, 10µl of the purified PCR product was mixed with 1µl of a 10mM dNTP mix (2.5mM each). Subsequently, 1x and 0.5U of the 10x polishing buffer and of the cloned *Pfu* DNA polymerase was added, respectively. Following gentle mixing, the reaction was overlayed with 20µl mineral oil, incubated at 72^{0}C for 30 minutes and subsequently stored at 4^{0}C until required.

3.3.7 Cloning of amplicon

A ligation reaction mixture was prepared, adding the following components (in order) in a 0.5ml microcentrifuge tube. Firstly, 10ng of the PCR-Script Amp SK (+) cloning vector was mixed with 1x of the PCR-Script 10x reaction buffer. Following this, 0.5µl of the 10mM rATP, 4µl of the flush ended PCR product, 5U of *Srf*I restriction enzyme and 4U of T4 DNA ligase was added. Sterile dH_2O to a final volume of 10µl was added. The ligation reaction was mixed gently and incubated for 16 hours at 14^{0}C, followed by heating at 65^{0}C for 10 minutes. The ligated DNA was transformed and following blue-white selection (Sambrook *et al.*, 1987), the DNA was extracted and sequenced.

3.3.8 Polyhedrin sequence alignment

A data set of 57-character-long aligned nontranslated leader sequences (Figure 3.2) was constructed (Zanotto *et al.*, 1993). Similarly, amino acid sequence data from 18 polyhedrin genes was aligned using

CLUSTAL W 1.74 (Higgens and Sharp, 1988), yielding an aligned data set consisting of 246-character-long sequences, including amino acids, ambiguities, missing characters and introduced gaps (Figure 3.3).

3.4 Results and Discussion

The TnSNPV polyhedrin gene was found by using an expression vector containing the AcMNPV polyhedrin gene to probe an *Eco*RI DNA library from TnSNPV by Southern blotting. This identified a positive signal from a 1.35 kb clone. End sequencing of this clone using universal sequencing primers found it to be similar to other baculovirus polyhedrin genes, however the 3' end was found to be truncated. To locate the missing sequence from the truncated end, inverse PCR primers were designed. By using inverse PCR on *Hind*III digested genomic DNA, it was possible to amplify the remaining portion of the gene. Sequencing of the positive *Eco*RI clone, subclones from it and the inverse PCR product was achieved using the sequencing strategy indicated in Figure 3.1.

Figure 3.4 shows the nucleotide sequence of a 1551-bp region of the TnSNPV DNA that contains the complete coding region for polyhedron. The sequences obtained were confirmed by at least two overlapping sequences. Two restriction endonuclease cleavage sites (*Dra*I and *Hind*III) had been previously located by restriction enzyme analysis and were correctly predicted by the nucleotide overlapping sequences. The sequencing data from the 1551 nucleotides sequenced suggests an ORF of 741 nucleotides encoding a putative protein of 247 amino acids with an estimated size of 28.89 kDa. The nontranslated leader sequence of TnSNPV was most similar to that of *Panolis flammae* MNPV (88%) and *Mamestra brassicae* MNPV (88%). Overall, elements in the noncoding leader sequence

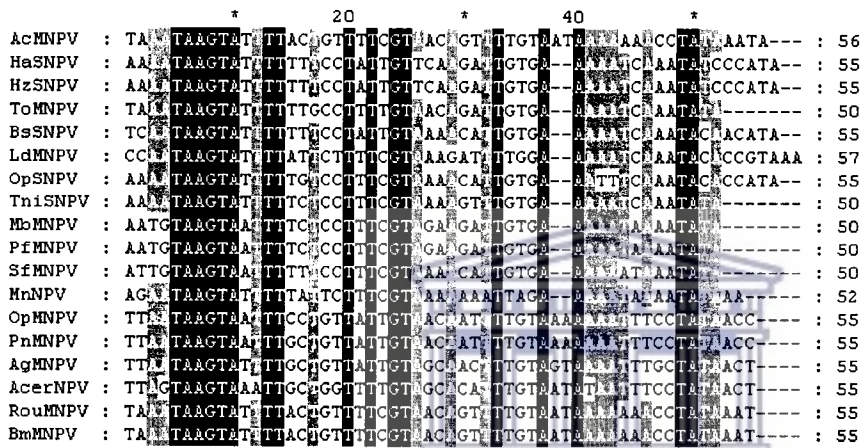


Figure 3.2: Alignment of baculovirus nontranslated upstream regions of 18 polyhedrin genes containing the transcriptional initiation site and promoter elements (Rohrmann, 1986; Zanotto *et al.*, 1993). AcMNPV was used as reference sequence. Dashes were introduced as gaps to maximise homology. Refer to Table 3.1 for abbreviations. Black shaded areas indicate 100% homology; deep grey 80% or more; light grey 60% or more.

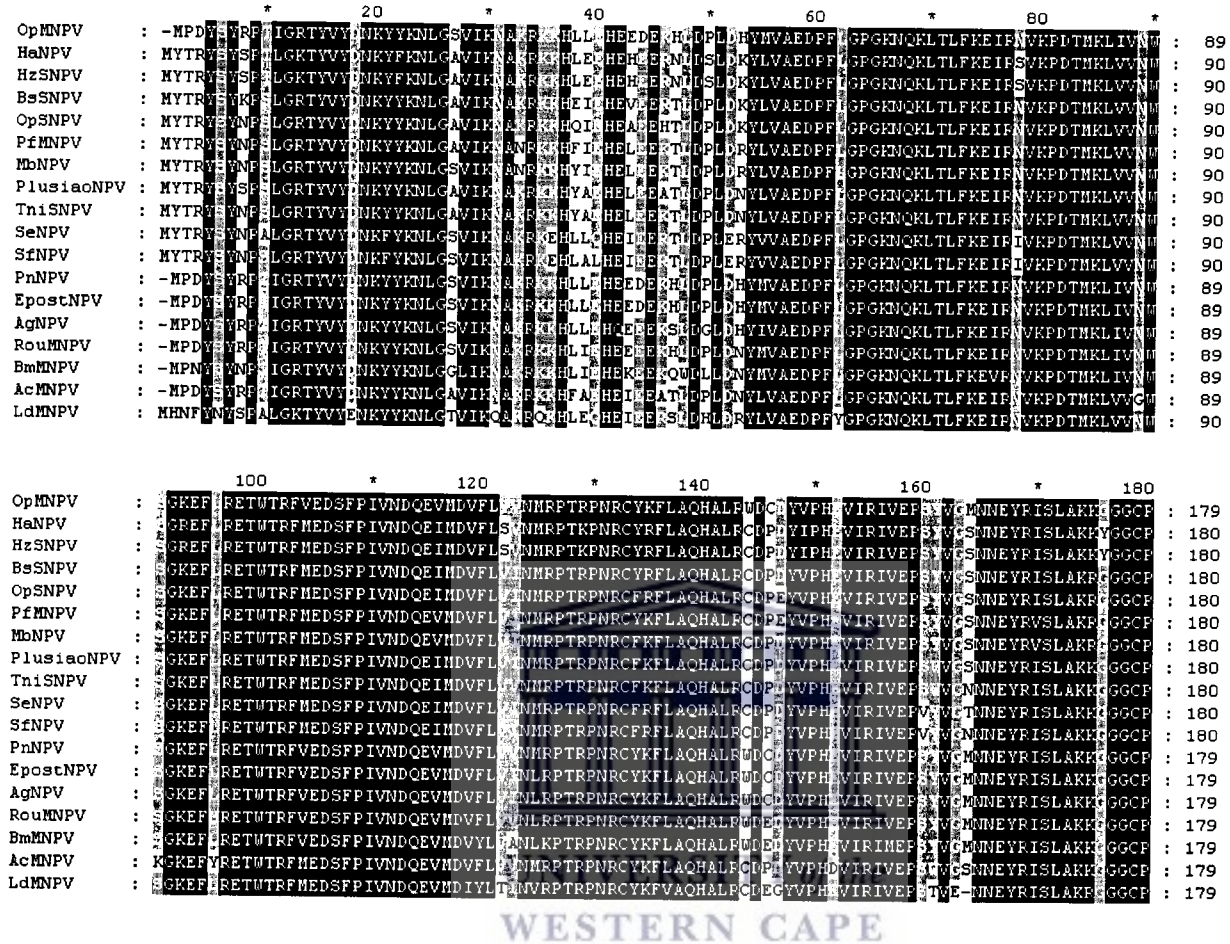


Figure 3.3: Alignment of the amino acid sequence of 18 baculovirus polyhedrin genes using CLUSTAL W 1.74. OpMNPV was used as reference group. Dots represent identical amino acids and dashes were introduced to maximize alignment of the sequences. Refer to Table 3.1 for abbreviations. Black shaded areas indicate 100% homology; deep grey 80% or more; light grey 60% or more.

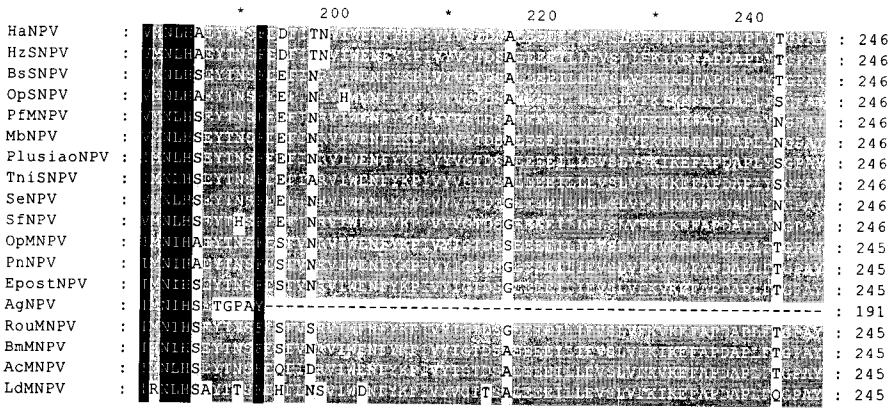


Figure 3.3. Continued

were found to be very similar to those of other polyhedrin genes (Figure 3.3). Similarities include an adenosine at position -3, with the exception of AcMNPV, and the possible duplication of the *TTNGTA* motif (Zanotto *et al.*, 1992). The noncoding leader sequence containing the baculovirus late transcription initiation motif (*TAAG*) is AT rich, consistent with other baculovirus late expression genes (Rohrmann, 1992). Comparison of the nucleotide sequence of the ORF shows the TnSNPV to be most closely related to the polyhedrin sequences of *Thysanoplusia oricalchea, Mamestra brassicae* NPV (strain Oxford) and *Panolis flammae* MNPV, with homology values in excess of 80%. Also, homology values for the prototype baculoviruses, *Orgyia pseudotsugata* SNPV and *Autographa californica* MNPV were in excess of 80% (results not shown). At the amino acid level the greatest homology was found to *Thysanoplusia oricalchea, Mamestra brassicae* NPV (strain Oxford), *Panolis flammae* MNPV, *Orgyia pseudotsugata* SNPV, and *Autographa californica* MNPV, with homologies of 98%, 94%, 93%, 93% and 92% respectively.

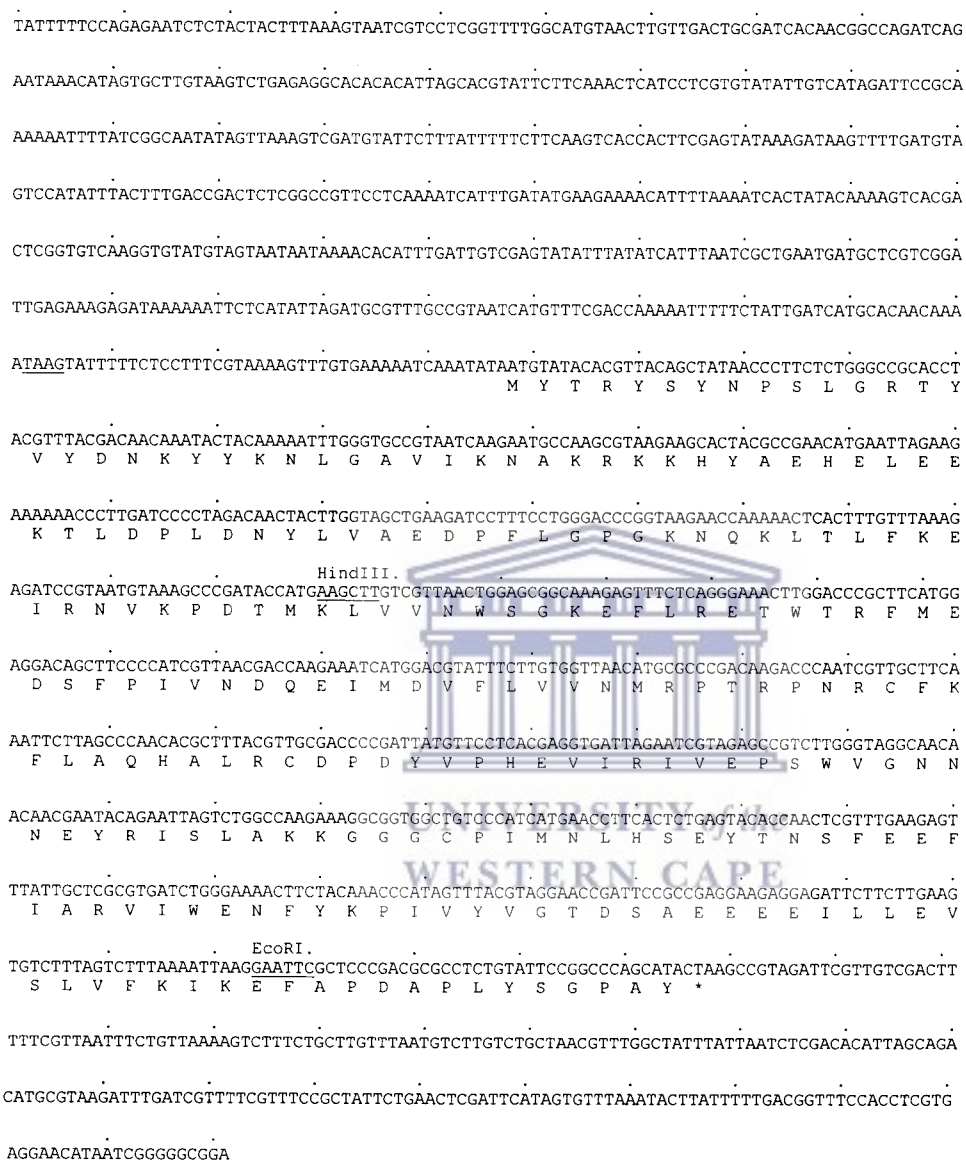


Figure 3.4: Nucleotide and predicted amino acid sequences of the TnSNPV polyhedrin gene and flanking regions. Restriction recognition sites for *Hind*III and *Eco*RI are indicated. The putative late translation initiation motif-core (TAAG) is underlined. No obvious poly(A) motif is present.

Table 3.1: NPVs used in this study.

Lepidopteran host	Abbreviation	Group*	Reference
Trichoplusia ni	Tn/TnSNPV		
Buzura supressaria	Bs/BsSNPV	I	Hu *et al.*, 1993
Panolis flammea	Pf/PfMNPV	II	Oakey *et al.*, 1989
Anticarsia gemmatalis	Ag/AgMNPV	I	Zanotto *et al.*, 1992
Spodoptera exigua	Se/SeMNPV	II	van Strien *et al.*, 1992
Spodoptera frugiperda	Sf/SfMNPV	II	Gonzalez *et al.*, 1989
Mammestra brassicae	Mb/MbMNPV	II	Cameron and Posee, 1989
Autographa californica	Ac/AcMNPV	I	Hooft Van Iddekinge *et al.*, 1983
Orgyia pseudotsugata	OpM/OpMNPV	I	
Bombyx mori	Bm/BmNPV	I	Iatrou *et al.*, 1985
Orgyia pseudotsugata	OpS/OpSNPV	II	Leisy *et al.*, 1986
Rachiplusia ou	Ro/RoNPV		Harrison and Bonning, 1999
Perina nuda	Pn/PnNPV		Chou *et al.*, Accession number
Epiphyas postvittana	Ep/EpNPV		Fairburn *et al.*, 1998
Heliothis armigera	Ha/HaSNPV	II	Chen *et al.*,
Helicoverpa zea	Hz/HzSNPV	II	Cowan *et al.*, 1994
Lymantria dispar	Ld/LdMNPV		Smith *et al.*, 1988
Thysanoplusia oricalchea	To/ToMNPV	II	

* Zanotto *et al.*, 1993; Cowan *et al.*, 1994

Attempts have been made to design a phylogenetic relationship between the different baculoviruses based on polyhedrin and ecdysteroid glucosyltransferase gene sequences (Zanotto *et al.*, 1992; Clarke *et al.*, 1996.). On this basis it has been proposed that baculoviruses fit into two phylogenetically diverse groups.

Group I is tentatively characterised by a N-terminal peptide sequence: MP(D/N)YS, a length of 245 amino acids and an obvious poly (A) terminal motif. Group II is characterised by a N-terminal peptide sequence MYT(R/P)YS, a length of 246 amino acids and only some members of this group lack a poly (A) tail. Group II has been further divided into two clades based on sequence similarity (Cowan *et al.*, 1994). Consistent with the characteristics of Group II, the TnSNPV was found to have a MYTRYS polypeptide sequence at the start of the ORF and a length of 246 amino acids, also there was no apparent poly (A) tail. Previous reports suggest that the appearance of a poly (A) tail could be a defining characteristic of Group I viruses (Zanotto *et al.*, 1992). Since no poly A tail was found here, it is further putative evidence for TnSNPV being in Group II.

To date baculovirus classification has relied heavily on the virus morphotype. Therefore, SNPV and MNPV were considered different subgenus of the genus NPV (Francki *et al.*, 1990). This is not supported by the polyhedrin phylogenies proposed to date. Additionally, GVs containing multiple occluded virions have been reported and BmNPV has been shown to revert between the SNPV and MNPV morphotypes, further discrediting the accepted classification scheme (Cowan *et al.*, 1994). Thus, in order to resolve baculovirus classification, more baculovirus genes need to be characterised to establish accurate phylogenetic relationships.

Chapter 4

Research Results

Identification and characterisation of the *Trichoplusia ni* single capsid nucleopolyhedrovirus *p10* gene

A portion of this chapter has been published as:

Fielding B.C. and Davison S. 2000. Identification and characterisation of the *Trichoplusia ni* single capsid nucleopolyhedrovirus *p10* gene. *Virus Genes* 20:2, 189-192.

The nucleotide data reported in this chapter have been submitted to GenBank and has been assigned the accession number AF150991.

Chapter 4

4.1 Abstract

The *p10* gene was identified and characterised from the *Trichoplusia ni* single capsid nucleopolyhedrovirus (TnSNPV). The p10 open reading frame (ORF) sequence was identified following sequencing of the ends of the *Eco*RI-G clone. Subsequent sequencing of an *Eco*RI-*Sma*I subclone identified the entire *p10* and a portion of a *p26* homologue. The p10 ORF of 264 basepairs (bps) encoded a predicted protein of 88 amino acids (aas) with M_r 9527 Da. The putative late transcription initiation motif (TAAG) was found upstream of the translation initiation codon at position -46. Downstream of the translation stop codon, a putative poly (A) signal was identified. The p10 amino acid sequence contained the three conserved domains reported for all other *p10* genes. The p10 amino acid sequence was most homologous (85% similarity and 67% identity) to that of *Buzura suppresaria* NPV p10 amino acid sequence.

4.2 Introduction

Trichoplusia ni single capsid nucleopolyhedrovirus (TnSNPV) belongs to the genus *Nucleopolyhedrovirus* within the family *Baculoviridae* (Francki *et al.*, 1991). This Group II baculovirus contains a closed circular, double stranded DNA genome approximately 160 kbs in size.

Baculovirus gene expression occurs in a temporally regulated cascade of four classes. These include immediate and delayed early genes as well as late and very late genes (Kool and Vlak, 1993). In the very late phase of gene expression, prior to occlusion body assembly, polyhedrin and *p10* are expressed in copious amounts (Wilson *et al.* 1995). Consequently, their strong promoters are frequently used in baculovirus expression vector systems (Miller, 1993). It has been used in the commercial expression of various important proteins, amongst others the expression of human α-interferon using BmNPV (Maeda *et al.*, 1985), as well as β-interferon (Smith *et al.*, 1983) and β-galactosidase (Pennock *et al.*, 1984) using AcMNPV.

P10, a relatively small peptide, affects virus replication in several ways, with specific regions within the peptide shown to confer unique functions (Wilson *et al.* 1995). The peptide forms part of the fibrillar structures observed in virus assembly (van der Wilk, 1987), ensuring greater occlusion body stability. Additionally, p10 plays a vital role in calyx formation and attachment (Wilson *et al.* 1995). Although p10 has been conserved throughout baculovirus evolution, the amino acid sequence has not been well conserved between different baculoviruses. Amino acid identity values between different baculoviruses range from 20% (Faktor *et al.*, 1997) to 94.6% (van Oers *et al.*, 1994). However, a higher similarity exits in the secondary protein structure and within specific domains (van Oers *et al.*, 1998; Faktor *et al.*,

1997), suggesting that *p10* evolved from an ancestor gene more rapidly than other baculovirus genes (Zuidema *et al.*, 1993).

Not much is known about the *TnSNPV*s' molecular arrangement and functioning, with only the polyhedrin gene sequenced to date (Fielding and Davison, 1999). This makes TnSNPV an attractive candidate for both genetic analysis and recombinant studies. Currently both a physical and functional genomic map is being constructed. In this study an *Eco*RI-SmaI subclone known to contain the *p10* gene, was sequenced. The *p10* gene was characterised as a prelude to using it in a baculovirus expression vector system. The predicted *p10* gene product is compared to other baculovirus p10 sequences. The p10 nucleotide and amino acid sequences were most homologous (60% and 67%, respectively) to that of *Buzura suppresaria* NPV. Downstream of the p10 ORF, we identified a partial p26 ORF homologue. Both of these ORFs are in the opposite orientation to the polyhedrin ORF reported previously (Fielding and Davison, 1999).

4.3 Methods

4.3.1 Virus isolation and DNA extraction

Methods used are those described previously (**Chapter 2** and Fielding and Davison, 1999).

4.3.2 Clone construction, location of the *p10* gene

Genomic DNA was used to construct an *Eco*RI library (Sambrook *et al.*, 1989). The library was used to create both partial functional and genomic maps of the TnSNPV genome. Random sequencing of the library using universal forward and reverse primers was performed ("sniff-sequencing"). A sequence

similarity search indicated that the *Eco*RI-G fragment contained a p10 ORF homologue. Subsequently, an *Eco*RI-*Sma*I subclone was created and sequenced in both directions. A complete putative p10 ORF and partial p26 ORF was identified (Figure 4.1).

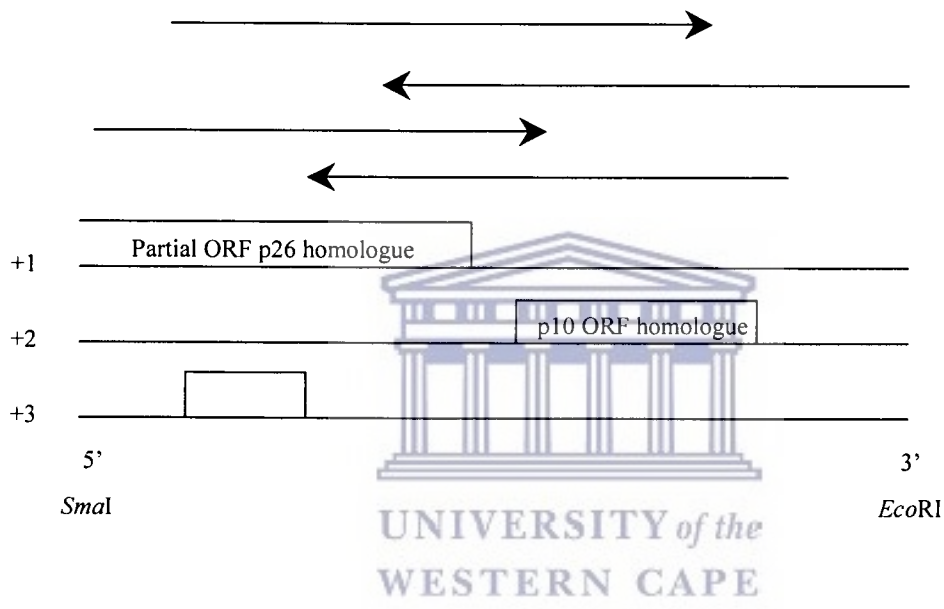


Figure 4.1: Sequencing strategy and ORF distribution of the TnSNPV *Eco*RI-*Sma*I subclone. The three reading frames for the top DNA strand are labelled 1, 2 and 3. The p10 ORF and the partial p26 ORF are indicated (not drawn to scale).

4.3.3 Homology searches

Nucleotide and amino acid sequences were compared with those in GenBank at the National Centre for Biotechnology by using the Advanced Blast Search Server (Altschul *et al.*, 1997). Additional sequence

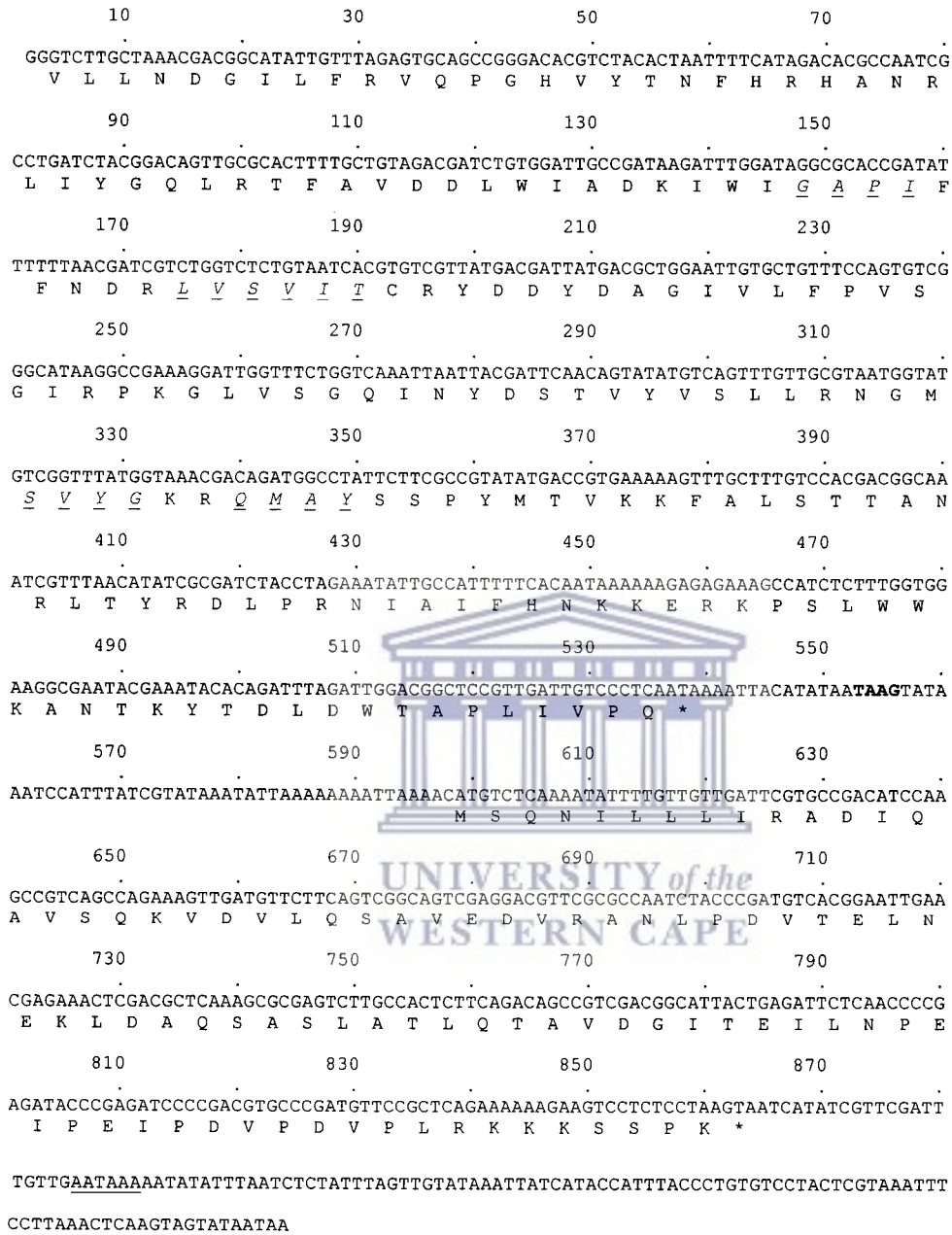


Figure 4.2: Nucleotide and amino acid sequences of *p10* (position 599-891), partial *p26* (1-538) and flanking regions. The *p10* poly (A) motif (AATAAA) is underlined and the late translation initiation motif-core (TAAG) is in bold. The conserved p26 amino acid sequence motifs are in italics-underlined.

analysis was done using the Genetics Computer Group Server. The putative TnSNPV *p10* gene was compared to other baculovirus *p10* genes using CLUSTAL W 1.74 (Higgens and Sharp, 1988) and homology shading was done using the GENEDOC software (Nicholas and Nicholas, 1997).

4.4 Results and Discussion

Analysis of the 983bp *Eco*RI-*Sma*I subclone suggests two ORFs separated by 50 bps. The sequence data containing the entire putative p10 ORF and a partial *p26* homologue is shown in Figure 4.2. Final sequences obtained were confirmed by at least two overlapping sequences. Similar to *Autographa californica* MNPV, *Choristoneura fumiferana* MNPV, *Orgyia pseudosogata* MNPV, *Bombyx mori* NPV, *Buzuria suppresaria* SNPV and *Spodoptera exigua* MNPV, the TnSNPV *p10* and *p26* genes are arranged in a collinear fashion (Rohrman, 1992). Additionally, both ORFs are in the opposite orientation to that predicted for the TnSNPV polyhedrin ORF (Fielding and Davison, 1999).

The p10 ORF of 264 bps, encodes a predicted protein of 88 amino acids with M_r 9527 Da. The putative late transcription initiation motif (TAAG) was found upstream of the translation initiation codon (ATG) at position -44. The 3'-noncoding region of the p10 ORF contained a putative poly (A) signal 20bps downstream of the translation stop codon. Consistent with the very late expression genes of other baculoviruses, the 5' non-coding leader sequence was found to be AT-rich (van Oers *et al.*, 1993). The TnSNPV p10 protein of 88 aas was compared to other p10 species ranging from 87-104 aas in length (Figure 4.3). The highest homology was to *Buzura suppresaria* NPV (identities 67% and similarity 85%). The primary amino acid sequence between different baculoviruses is not well conserved. However, evolutionary pressure has preserved the structure of the protein and hence its function. Three

```
Ldnpv  msqnillvir qdisnlsdqv talqgav--- ----ddvran .........k
Sinpv  .......... .......... .......--- ----...... ..........
Slnpv  .......... ...k...... .......--- ----...... ..........
Bsnpv  .......... s..ka.dtk. ....qq.tdv qqqit..qs. ...i..l...
Senpv  .......l.. a..kavdek. d...q..--- ----n..s.. ...ts.lsa.
Tnnpv  .......l.. a..qav.qk. dv.qsa.--- ----e..... ......l.e.

Ldnpv  ldaqnaqlvs leasneaist lvqslseavq nitdiltpei pdlpipnppl
Sinpv  .......... .......... .......... .......... ..........
Slnpv  .......... .......... .......... ........d. ......d...
Bsnpv  ....s.t.tn .qtiv....d ilnpeipdlp dvpglrktgt glkk------
Senpv  ....att.dt ivtqvnn.nd vlnpdlpd.p gnlqkqqqqk ksnkk-----
Tnnpv  ....s.s.at .qtavdg.te ilnpeipeip dvp.vplrkk ksspk-----

Ldnpv  g-kknnggin kk
Sinpv   .-........ ..
Slnpv  .n......v. ..
Bsnpv  ---------- --
Senpv  ---------- --
Tnnpv  ---------- --
```

Figure 4.3: Alignment of the amino acid sequences of TnSNPV p10 to five other NPVs. The sequences aligned were: Tnnpv, *Trichoplusia ni* SNPV; Senpv, *Spodoptera exigua* NPV (Zuidema *et al.,* 1993); Bsnpv, *Buzura suppresaria* NPV (Van Oers *et al.,* 1998); Sinpv, *Spodoptera littoralis* NPV (Faktor *et al.,* 1997); Slnpv, *Spodoptera litura* NPV (Wei *et al.*, unpublished); Ldnpv, *Lymantria dispar* NPV (Kuzio *et al.,* 1999). Dashes were introduced to align sequences and dots represent identical amino acids.

conserved p10 amino acid sequence domains have been identified (Figure 4.4). These included an N-terminal coiled-coil structure that is involved in the intramolecular interaction leading to the aggregation of p10 (Wilson *et al.* 1995), a basic domain at the C-terminus implicated in the alignment of p10 aggregates into fibrillar structures (Van Oers *et al.*, 1993) and a Proline rich domain that plays a role in

the polyhedra release from the infected nucleus (Zuidema *et al.,* 1993; Van Oers *et al.*, 1993). The partial p26 amino acid sequence was most homologous to that of *Buzura supressaria* NPV (identity 44% and similarity 67%).

M S Q N I L L L I R A D I Q A V S Q K V D V L Q S A V E

D V R A N L P D V T E L N E K L D A Q S A S L A T L Q T

A V D G I T E I L N **P E I P E I P D V P D V P** L *R K K K*

S S P *K*

Figure 4.4: Conserved domains identified in the TnSNPV p10 amino acid sequence. The heptad-repeat sequences in the amino-terminal domain are underlined, the proline-rich domain is in bold and the positively charged residues in the C-terminal are in italics. The conserved domains are predicted by comparing the BsSNPV and TnSNPV amino acid sequences.

Conserved p26 amino acid sequence motifs that were found in other NPVs were also identified here. These included GAPI, SVYG, LVSV(I/V)T and Q(M/LA/P)Y (Rohrman, 1992). The exact function of *p26* has not been determined (van Oers *et al.*, 1998). However, it has been shown to be non-essential for *in vitro* replication (Rodems and Friesen, 1993).

Hydrophilicity plots (Figure 4.5) showed a high degree of similarity between the p10 proteins compared.

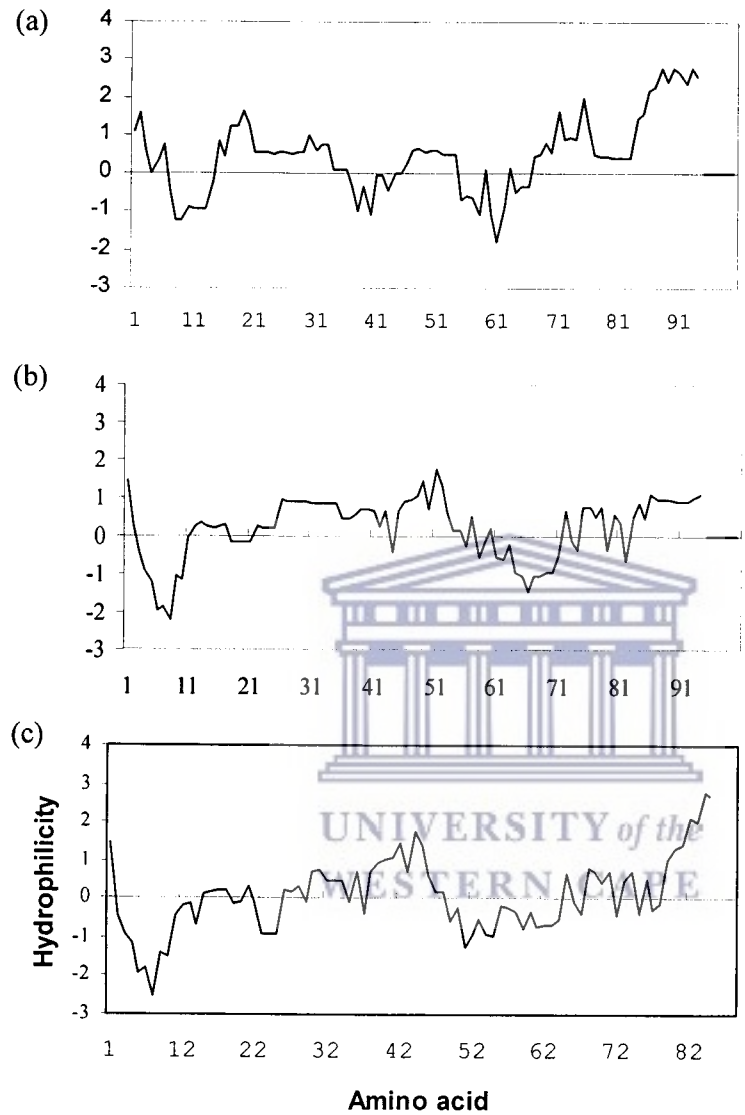


Figure 4.5: Hydrophilicity plots of the p10 polypeptides of a) AcMNPV, b) BsSNPV and c) TnSNPV. The plots were constructed using the Kyte and Doolittle (1982) algorithm from the PEPTIDESTRUCTURE program of GCG. Values above the zero line indicate hydrophilic residues and values below the line indicate hydrophobic residues (van Oers *et al.*, 1993).

A weakly hydrophobic region was found at the N-terminus of the polypeptides. This region could possibly be involved in bundling or cross-linking (Vlak et al., 1988). The C-terminus was found to be highly hydrophilic and contained several basic amino acid residues. This carboxy terminus is thought to be exposed at the surface of the protein (Van Oers *et al.*, 1993). Also, the C-terminal half of the peptides was proline rich. Thus, even though the primary amino acid sequences of p10 are poorly conserved, the structure of the polypeptides is fairly conserved. To our knowledge, this is only the second SNPV *p10* gene sequenced. This supports the implication that the *p10* gene is indeed preserved in all NPVs (Rohrman, 1992).

Chapter 5

Research Results

The genetic organisation of a 2966 basepair DNA fragment and phylogeny of a single capsid nucleopolyhedrovirus isolated from *Trichoplusia ni*

The contents of this chapter will be submitted for publication as:

Fielding B.C. and Davison S. The genetic organisation of a 2.9kb DNA fragment of a single capsid nucleopolyhedrovirus isolated from *Trichoplusia ni*

The nucleotide data reported in this chapter have been submitted to GenBank and has been assigned the accession number AF358416

5.1 Abstract

The nucleotide sequence of a 2966 basepairs (bps) fragment of the *Trichoplusia ni* single capsid nucleopolyhedrovirus (TnSNPV) was determined and mapped. The clone was found to contain five open reading frames (ORFs) homologous to baculovirus genes, including *p26*, fibrillin (*p10*), SeORF128, late expression factor 6 (*lef-6*) and the C-terminal portion of *p74*, on either strand of DNA. The arrangement of the ORFs in the fragment was compared to that of the prototype virus *Autographa californica* multiple capsid NPV (AcMNPV), *Orgyia pseudosogata* MNPV (OpMNPV), *Spodoptera exigua* MNPV (SeMNPV) and *Lymantria dispar* MNPV (LdMNPV). TnSNPV has previously been putatively identified as a member of the Group II NPVs. To confirm the phylogenetic relationship of TnSNPV, the amino acid sequences of *p26*, partial *p74* and *lef-6* were analysed and compared to a phylogenetic tree generated from occlusion body amino acid sequences. The peptide sequences were aligned for positional homology and trees were calculated using distance and maximum likelihood methods. Predicted amino acid sequences for the ORFs were compared and homology values of 12-57% were observed. Clustering and arrangement of the TnSNPV genes were similar to the clustering reported for SeMNPV. Phylogenetic analysis supported three Subclades in Group II, namely A, B and C, with TnSNPV classified in Group IIA.

5.2 Introduction

Two genera make up the family Baculoviridae, the *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Hu *et al.*, 1997), all of which infect arthropods (Volkman *et al.*, 1995).

GVs have been isolated exclusively from lepidopteran larvae (Blissard and Rohrman, 1990; Tweeten *et al.*, 1981). Although, GVs infecting different Lepidoptera are structurally complex, all are very similar in morphological characteristics (Stairs *et al.*, 1966). NPVs infect more than 600 insect species, with a single virus isolate normally restricted to one host or a few closely related species (Blissard and Rohrmann, 1990). A few NPVs infect crustaceans, with the vast majority infecting insects (Washburn *et al.*, 1999). Whereas SNPVs have been isolated from *Hymenoptera*, *Lepidoptera*, *Diptera*, *Coleoptera*, *Trichoptera* and *Siphonoptera* (Kool *et al.*, 1995; King and Possee, 1994), MNPVs have only been isolated from the evolutionary youngest insect order, the *Lepidoptera* (Washburn *et al.*, 1999). This would suggest that MNPVs originated from the SNPVs and then radiated along with their hosts through evolutionary time (Rohrmann, 1986).

Although the family Baculoviridae consists of more than 600 viruses, only nineteen have received species status, including 15 assigned NPVs and four GVs (Murphy *et al.*, 1995). AcMNPV the prototype baculovirus, contains a closed circular double stranded DNA genome approximately 128kb in size (Friesen and Miller, 1987). NPV replication occurs in the nuclei of susceptible host-cells and viral genes are expressed in a temporally regulated cascade of events (Friesen and Miller, 1986). The transcription of AcMNPV DNA is characterised by the synthesis of numerous overlapping, polyadenylated RNAs (Oellig *et al.*, 1987; Friesen and Miller, 1987). Transcripts have been classified

as immediate and delayed early, as well as late and very late (Friesen and Miller, 1989). While early gene expression precedes viral DNA replication, late gene expression occurs after or concurrently with the start of viral DNA replication (Blissard and Rohrmann, 1990; Friesen and Miller, 1986). Moreover, whereas early transcripts are regulated by a host RNA polymerase II, late transcripts are regulated by a virus-induced RNA polymerase (Hardin and Weaver, 1990; Fuchs *et al.*, 1983). *Cis*- and *trans*-acting elements are involved in baculovirus DNA replication (Kool *et al.*, 1994). These *cis*-acting elements include unique non-homologous regions (Lu *et al.*, 1997) and palindromic repeats in homologous regions (*hr*), dispersed throughout the baculovirus genome. Five *trans*-acting elements are thought to be involved in DNA replication. These include DNA polymerase, DNA helicase, and three late expression factors (*lef*1-3) (Chen *et al.*, 1999).

Both AcMNPV and another baculovirus, OpMNPV, are well characterised, with the complete genomes sequenced (Ahrens *et al.*, 1997). Comparative sequence analysis of specific regions of the AcMNPV and OpMNPV genomes, have shown major differences in gene content and arrangement (Blissard and Rohrmann, 1990; Theilmann and Steward, 1992). Although the organisation of baculovirus genomes is studied extensively, little is known about the genetic relationships between different baculoviruses (Jin *et al.*, 1997). Recently, however, the complete sequencing of five MNPV genomes has elucidated important information about the relationships between the genome organisation and arrangement of genes of MNPVs. In order to examine the relationship between SNPVs and MNPVs, more complete genomic sequence data from SNPVs are needed.

Baculoviruses not only infect several different insect orders, but also distantly related arthropods such as

marine crustaceans (Crawford and Granados, 1982; Zanotto *et al.*, 1993). This could possibly indicate a prehistoric association between baculoviruses and invertebrates (Zanotto *et al.*, 1993). Baculovirus classification has been based on gross morphology (Francki *et al.*, 1991) and their nomenclature on the host from which they were first isolated (Tinsley and Kelley, 1985). This has led to inaccurate classification and nomenclature, with virtually identical isolates being classified as separate subgenera. A thorough understanding of baculovirus phylogenetic relationships could be used to resolve the nomenclature of baculoviruses. Also, it could be important in the future development of biological pesticides and eukaryotic expression systems based on intertypic recombinants (Zanotto *et al.*, 1993). Baculovirus phylogenetic relationships have been proposed using the DNA and protein sequences from polyhedrin (Zanotto *et al.*, 1993; Cowan *et al.*, 1994), *egt* (Hu *et al.*, 1997; Barrett *et al.*, 1995), DNA polymerase (Bulach *et al.*, 1999), *gp37* (Jin *et al.*, 1999), *gp41* (Liu and Maruniak, 1999) and *lef2* (Chen *et al.*, 1999), too name but a few. Based on analysis of these sequences, NPVs have been divided into two apparently distinct branches, designated Group I and Group II (Zanotto *et al.*, 1993). However, as yet, many NPVs are still to be classified.

Trichoplusia ni single capsid nuclear polyhedrosis virus, a member of the family Baculoviridae, possesses a genome about 159kb in size. The virus is not well characterised with only the polyhedrin (Fielding and Davison, 1999), p10 (Fielding and Davison, 2000) and *ie*-1 (Wang *et al.*, in press) genes sequenced to date. To gain some insight into the reason why the TnSNPV genome is 30kbs larger than AcMNPV, genome libraries are being sequenced and mapped. To date, only multiple nuclear polyhedrosis virus (MNPVs) genomes have been completely sequenced and analysed. This limits the understanding about the relationship between the genomic organisation of MNPVs and SNPVs. In this

chapter the sequence of a 2.97kb fragment of the TnSNPV genome was determined and analysed. Analysis of this fragment was used to define differences between baculoviruses and to provide information for the further investigation of TnSNPV phylogeny.

Based on structural similarities of the polyhedrin gene, TnSNPV has putatively been classified as a Group II baculovirus (Fielding and Davison, 1999). However, in order to assign a baculovirus to either Group I or Group II, numerous genes need to be analysed (Zanotto *et al.*, 1993). In this chapter, four different TnSNPV genes are compared to baculovirus homologous and a phylogenetic relationship is proposed. Phylogenetic analysis identified TnSNPV as a Group II virus, specifically of the Subclade IIA.

5.3 Methods

TnSNPV was propagated in field-collected late fourth or early fifth instar *Trichoplusia ni* larvae. Larvae were reared on an artificial lepidoptera diet (Appendix), with a synthetic 12:12 hour day-night cycle at 65% humidity. TnSNPV was purified as described previously (**Chapter 2**) and purified OBs were used to extract genomic DNA, which was digested with various enzymes and analysed by agarose gel electrophoresis (Sambrook *et al.*, 1989).

5.3.1 Cloning and Sequencing

Genomic DNA was digested with *Pst*I (Boehringer Mannheim) and shotgun-cloned into the compatible restriction sites of pBluescript SK^+ to construct a partial library (Sambrook *et al.*, 1989). A 2966bp *Pst*I-*Hind*III subclone was selected for sequencing. Automated sequencing of the end-termini revealed

the presence of the C-terminal portion of the TnSNPV *p74* gene and a portion of a *lef*-6 homologue. Subsequently, subclones were constructed and sequenced using universal primers with a Pharmacia ALF/Express automated sequencer.

5.3.2 Computer analysis

Sequence analysis was performed using the GCG computer program. Conceptual amino acid sequences were compared with sequence homologues at GenBank/EMBL using the Advanced Blast Search Server (Altschul *et al.*, 1997). Sequence alignments were done using CLUSTAL W 1.74 (Higgens and Sharp, 1988) or PILEUP at GCG and GENEDOC software was used for homology shading of the aligned amino acid sequences.

5.3.3 Phylogenetic analysis

Polyhedrin

Amino acid sequence data from thirty-three occlusion body proteins were aligned using CLUSTAL X 1.8 (Thompson *et al.*, 1997), yielding an aligned data set consisting of 246-character-long sequences, including amino acids, ambiguities, missing characters and introduced gaps. Phylogenetic trees were obtained using distance matrix and neighbour joining (Saitou and Nei, 1987) methods from CLUSTAL X 1.8. The robustness of all tree data sets was tested using the bootstrap method (Efron, 1982) and trees were viewed using the tree drawing software NJ-plotWIN95.

The polyhedrin amino acid sequences from the following baculoviruses were used in the phylogenetic analysis: *Autographa californica* MNPV (Hooft Van Iddekinge *et al.*, 1983), *Bombyx mori* NPV (Iatrou

et al., 1985), *Orgyia pseudotsugata* SNPV (Leisy *et al.*, 1986), *Anticarsia gemmatalis* NPV (Zanotto *et al.*, 1992), *Mamestra brassicae* NPV (Cameron and Possee, 1989), *Panolis flammea* NPV (Oakey *et al.*, 1989), *Spodoptera frugiperda* MNPV (Gonzalez *et al.*, 1989), *Spodoptera exigua* NPV (van Strien *et al.*, 1992), *Orgyia pseudotsugata* MNPV (Leisy *et al.*, 1986), *Rachiplusia ou* NPV (Harrison and Bonning, 1999), *Perina nuda* MNPV (Chou *et al.*, unpublished), *Amsacta albistriga* NPV (Premkumar and Mathavan, unpublished), *Epiphyas postvittana* NPV (Fairbairn *et al.*, 1998), *Heliothis armigera* SNPV (Chen *et al.*, unpublished), *Helicoverpa zea* SNPV (Cowan *et al.*, 1994), *Buzura suppressaria* SNPV (Hu *et al.*, 1993), *Lymantria dispar* MNPV (Smith *et al.*, 1988), *Choristoneura fumiferana* NPV (Rieth *et al.*, unpublished), *Hyphantria cunea* NPV (Lee *et al.*, 1992), *Malacosoma neustria* NPV (Jankevica *et al.*, unpublished), *Spodoptera littoralis* NPV (Croizier and Croizier, 1994), *Thysanoplusia orichalcea* MNPV (Cheng and Carner, 1999), *Malacosoma disstria* NPV (Erlandson *et al.*, unpublished), *Wiseana signata* NPV (Sadler *et al.*, 1999), *Ectropis oblique* NPV (Zhang *et al.*, unpublished), *Choristoneura rosaceana* NPV (Lucarotti and Morin, unpublished), *Mamestra configurata* NPV (Li *et al*, 1997), *Spodoptera litura* (Bulach *et al.*, unpublished), *Anagrapha falcifera* (Frederici *et al.*, 1996), *Neodipreon sertifer* (Zanotto *et al.*, 1993), *Trichoplusia ni* GV (Akiyoshi *et al.*, 1985) and *Agrotis segetum* GV (Kozlov *et al.*, 1992), *Choristoneura fumiferana* GV (Bah *et al.*, 1997).

Lef-6, *p26* and *p74*

The conceptual amino acid data from the available genes were aligned using PileUp at GCG, yielding aligned data sets consisting of amino acids, ambiguities, missing characters and introduced gaps. Unrooted trees were estimated using the least squares tree optimisation procedure (FITCH) and protein sequence distances were determined by PROTDIST from PHYLIP version 3.5c (Felsenstein, 1986).

Table 5.1: *Baculoviridae* used in this study

Lepidoptera species	S/M morphotype	Abbreviaiton used	Group[β]
Amsacta albistriga		AalbNPV	
Anagrapha falcifera		AfNPV	
Anticarsia gemmatalis	M	AgMNPV	I
Autographa californica	M	AcMNPV	I
Bombyx mori		BmNPV	I
Buzura suppressaria	S	BuzuSNPV	II
Choristoneura fumiferana	M	CfMNPV	I
Choristoneura rosaceana		CrosNPV	
Epiphyas postvittana		EpostNPV	
Ectropis obliqua		EoNPV	II
Helicoverpa zea	S	HzSNPV	II
Heliothis armigera	S	HaSNPV	II
Hyphantria cunea		HcuNPV	I
Lymantria dispar	M	LdMNPV	II
Malacosoma neustria		MnNMPV	II
Malacosoma disstria		MdisNPV	
Mamestra brassicae	M	MbMNPV	II
Mamestra configurata		MconNPV	II
Orgyia pseudotsugata	S	OpSNPV	I
Orgyia pseudotsugata	M	OpMNPV	I
Panolis flammea	M	PfMNPV	II
Perina nuda	M	PnMNPV	I
Rachiplusia ou		RouNPV	
Spodoptera littoralis		SlNPV	II
Spodoptera frugiperda	M	SfMNPV	II
Spodoptera litura		SlitNPV	II
Spodoptera exigua	M	SeMNPV	II
Trichoplusia ni	S	TnSNPV	II
Thysanoplusia orichalcea	M	ToMNPV	II
Wiseana signata		WsNPV	II
Neodoprion sertifer[α]	S	NsSNPV	N/A
Trichoplusia ni	N/A	TniGV	N/A
Agrotis segmentum	N/A	AsGV	N/A
Choristoneura fumiferana	N/A	CfGV	N/A

α Hymenopteran NPV used as outgroup; β Lepidopteran NPV group defined by Zanotto *et al.* (1993)

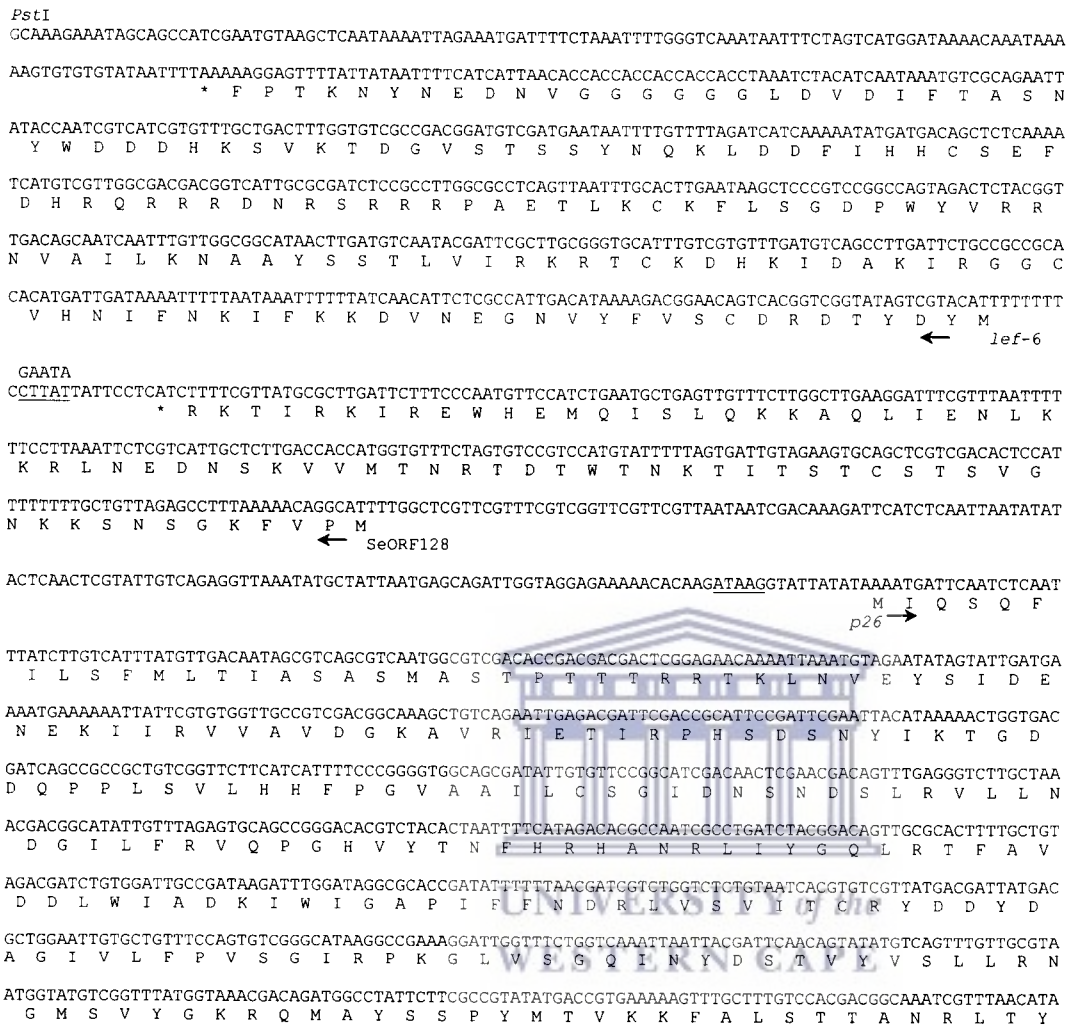


Figure 5.1: Nucleotide sequence of the TnSNPV genomic fragment. The predicted amino acid sequence is represented by the one-letter code designation below the nucleotide sequence. The ORFs and their direction of transcription are shown. Putative transcriptional motifs for early genes (TATA and CAGT), TAAG for late gene transcription and putative poly-A motifs are underlined. Selected restriction endonuclease sites are indicated. Signals on the complementary strand of the DNA are underlined and their complementary sequences are shown.

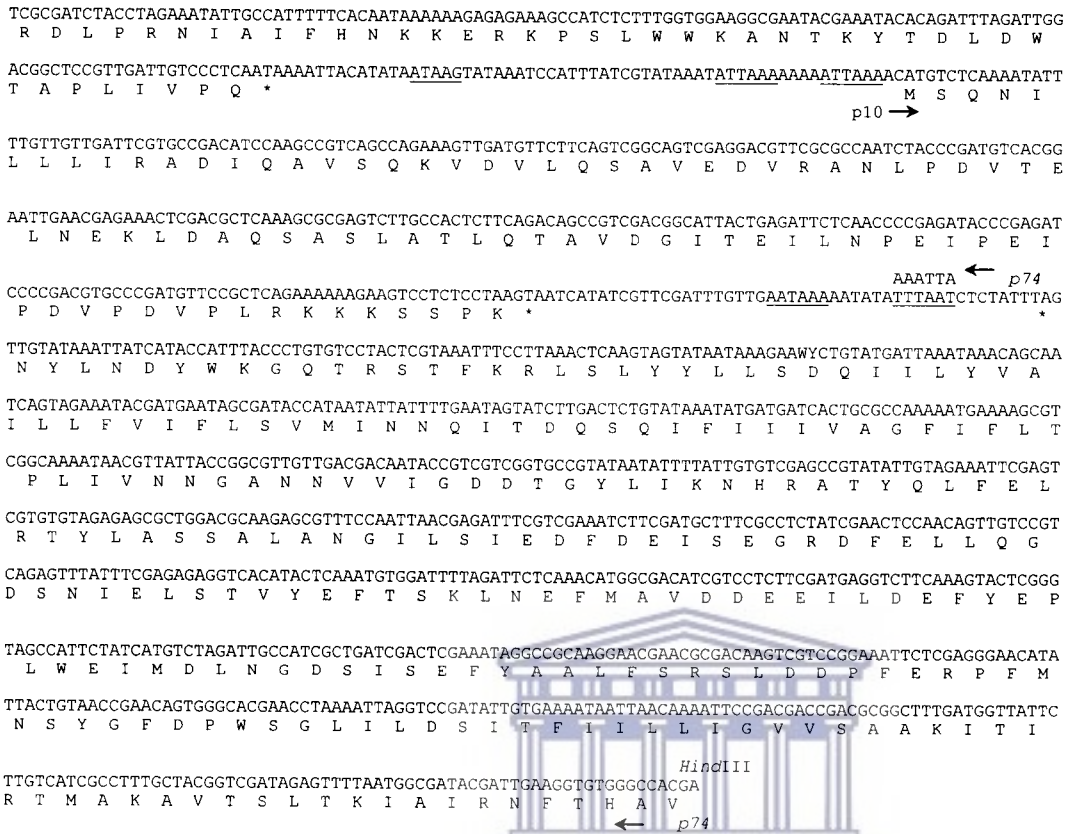


Figure 5.1: Continued

The robustness of the trees was tested using the bootstrap method (Efron, 1982) from SEQBOOT. Unless stated otherwise, the *p26*, *lef*-6, SeORF-128 and *p74* sequences gene sequences were taken from Ayres *et al.* (1994), Gomi *et al.* (1999), Ijkel *et al.* (1999), Kuzio *et al.* (1999) and Ahrens *et al.* (1997).

5.4 Results and Discussion

5.4.1 Sequence analysis

Five ORFs homologous to baculovirus proteins were identified within the fragment. These included *p26*, SeORF-128, *p10*, *lef*-6 and the C-terminal portion of *p74*. The nucleotide and putative amino acid sequences of the ORFs are presented in Figure 5.1.

lef-6

Lef-6 is one of eighteen genes thought to be essential for DNA replication of both AcMNPV and OpMNPV (Lu and Miller, 1995; Pari *et al.*, 1993). The gene has been shown to be involved in the expression of both the late major capsid protein (*vp39*) and the very late protein, polyhedrin (*polh*). It is believed that *lef*-6 functions either as a *trans*-acting transcriptional or translational activator, or as a primary or accessory replication factor. The AcMNPV *lef*-6 ORF contains a possible nuclear targeting sequence motif, *KRPRR*. Furthermore, AcMNPV *lef*-6 and Ac-*iap* have been shown to be cotranscribed (Passarelli and Miller, 1994).

Thus far, the TnSNPV *lef*-6 homologue encoding for a potential protein 158 amino acids in length, is the first SNPV *lef*-6 gene identified. A potential late translation initiation motif (*ATAAG*) was located 13nt upstream of the putative translation start codon (*ATG*). No obvious polyadenylation signal was found downstream of the putative translation stop codon (*TAA*). The 474 nt ORF encodes for a putative protein with M_r 18.29 kDa. Overall, with the exception of AcMNPV and BmNPV, low amino acid sequence identity was observed between the different baculoviruses compared (Table 5.2). The conceptual TnSNPV *lef*-6 amino acid sequence was most homologous to SeMNPV, with 35% sequence

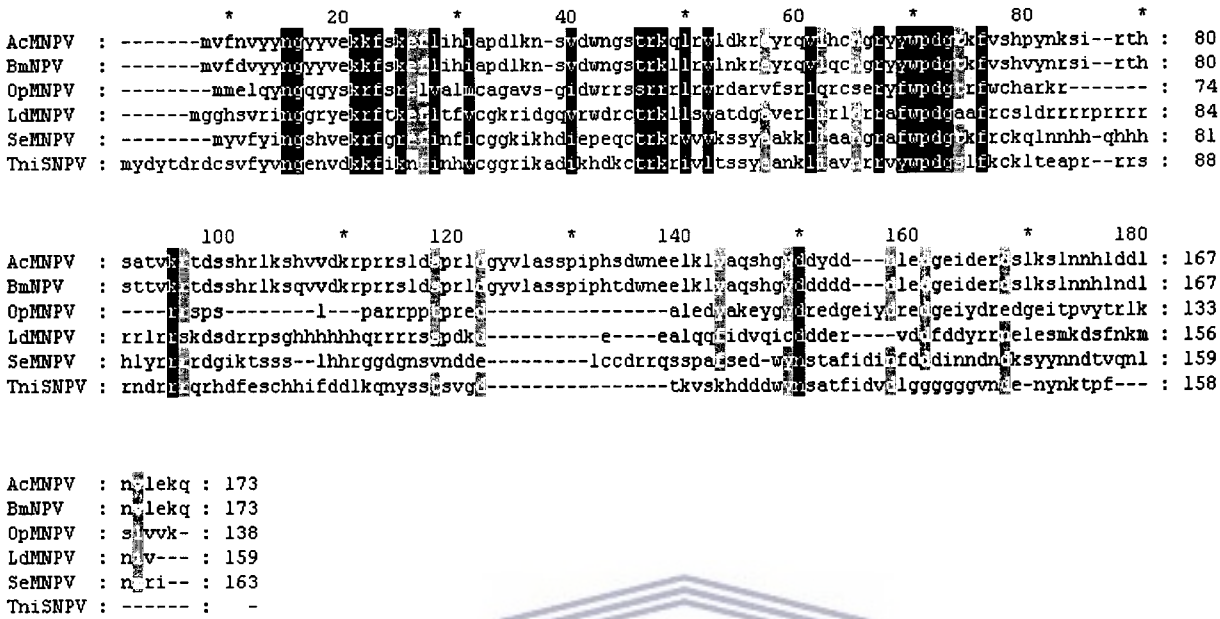


Figure 5.2. Alignment of the deduced amino acid sequences of putative *lef*-6 genes from six baculoviruses was done using the PileUp program at GCG. Dashes were introduced to align the sequences. Black shaded areas indicate 100% homology; deep grey 80% or more; light grey 60% or more. Refer to the Materials and Method section for abbreviations.

identity and 50% similarity. Four conserved amino acid sequence motifs appear to be present: NG, K(R/K)F, TRK and (F/Y)WPDG. However, the possible AcMNPV nuclear targeting sequence mentioned earlier, is not present in the TnSNPV LEF-6 protein (Figure 5.2).

Table 5.2. Identity and similarity (%) values of six putative *lef*-6 genes

	SeMNPV	LdMNPV	AcMNPV	BmNPV	OpMNPV
TnSNPV	**35** 50	**23** 40	**18** 29	**17** 29	**16** 28
SeMNPV		**23** 36	**24** 39	**23** 40	**17** 31
LdMNPV			**19** 33	**20** 34	**20** 32
AcMNPV				**93** 97	**20** 36
BmNPV					**20** 36

for abbreviations see Materials and Methods section

p74

During the baculovirus replication cycle, two phenotypically distinct viruses are produced (Volkman *et al.*, 1976). The one phenotype, viral occlusion bodies (OBs), is responsible for the horizontal transmission of virus between insects. After ingestion, OBs are dissolved by the alkaline midgut, releasing polyhedra-derived virions (PDVs). Polypeptide p74 appears to be important for the successful infection of insects, but not for cell culture (Kuzio *et al.*, 1989). Peptide p74 is not glycosylated and it has been shown that expression of the AcMNPV *p74* gene is essential for the production of infectiousOBs (Hill *et al.*, 1993; Kuzio *et al.*, 1989). The peptide appears to be a structural polypeptide of PDVs, most probably associated with the outside surface of the virion envelope (Faulkner *et al.*, 1997). Following ingestion, infectious OBs are dissolved by the alkaline insect midgut,

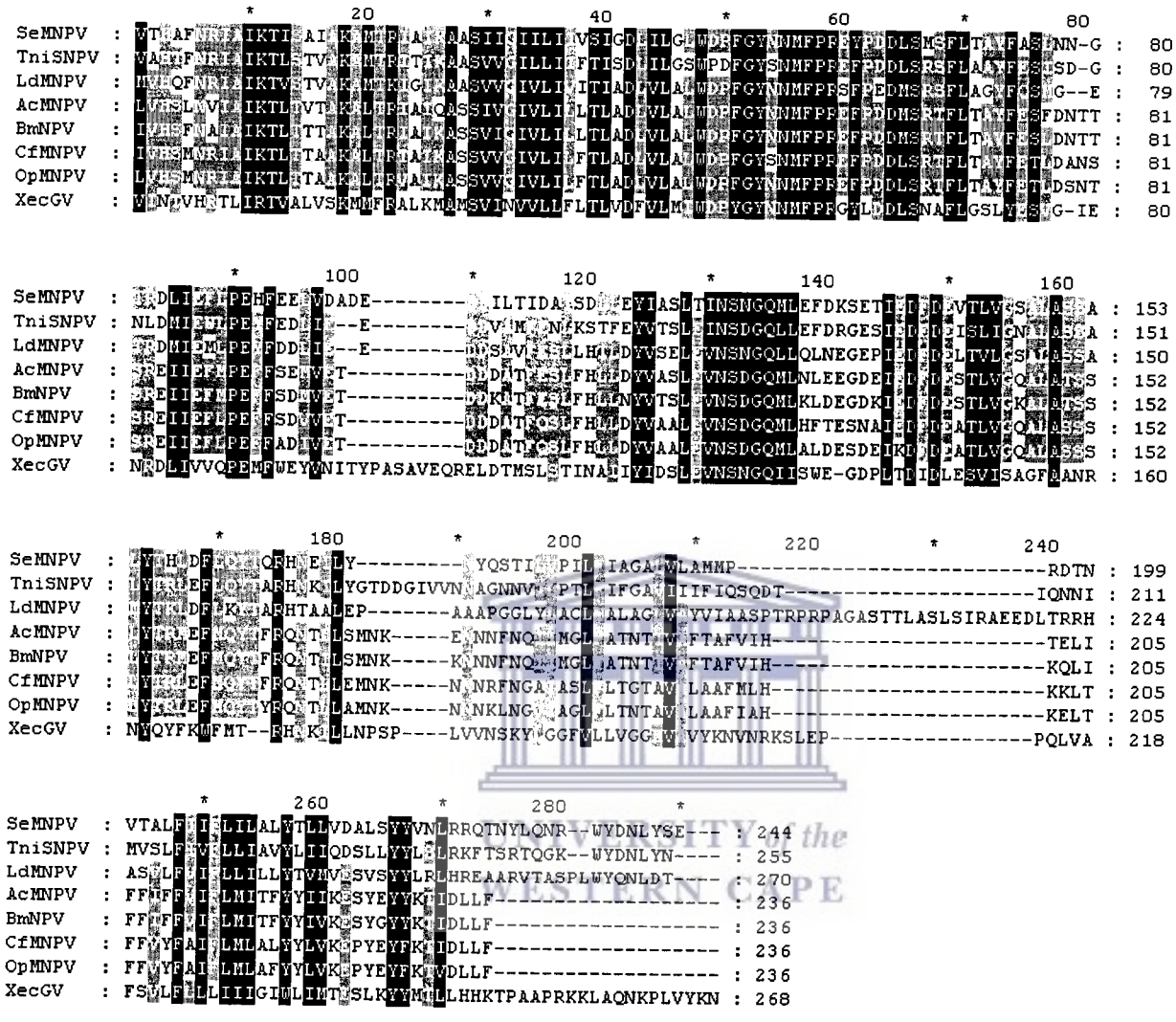


Figure 5.3. Alignment of the deduced amino acid sequences of putative baculovirus *p74* genes was done using the PileUp program at GCG. Dashes were introduced to align the sequences. Black shaded areas indicate 100% homology; deep grey 80% or more; light grey 60% or more. Refer to the Materials and Method section for abbreviations.

releasing polyhedra-derived virions (PDVs). Although it is expressed at low levels late in the AcMNPV infection cycle, no conventional late transcription initiation motif (*TAAG*) is present (Rohrmann, 1992).

Table 5.3. Identity and similarity (%) values of eight partial *p74* genes

	TnSNPV	LdMNPV	AcMNPV	BmNPV	CfMNPV	OpMNPV	XecGV
SeMNPV	**57** 76	**44** 65	**43** 66	**44** 66	**46** 64	**45** 66	26 50
TnSNPV		**47** 65	**43** 65	**46** 66	**45** 65	**43** 65	**26** 50
LdMNPV			**42** 62	**43** 62	**42** 60	**42** 60	**26** 47
AcMNPV				**90** 97	**76** 89	**77** 91	**26** 48
BmNPV					**76** 88	**77** 91	**26** 47
CfMNPV						**89** 96	**23** 48
OpMNPV							**24** 48

*values in bold indicate identity

The C-terminal portion of a TnSNPV *p74* homologue was identified and compared to baculovirus homologues (refer to Methods); partial *p74* genes of CfMNPV (Hill *et al.*, 1993) and *Xestia c-nigrum* (Xec) GV (Hayakawa *et al.*, 1999) were included in the analysis. The partial TnSNPV gene was most homologous to the SeMNPV *p74* gene (57% identity and 76% similarity). As expected, the GV *p74* homologue showed relatively low identity values (23%-26%) to the NPV *p74* genes (Table 5.3).

TnSNPV *p74* is in the opposite orientation to the *p10* homologue identified. A possible poly-A motif was identified 7nt downstream of the putative stop codon (TAA). Several conserved motifs were observed in both the NPV and GV *p74* homologues (black shaded areas in Figure 5.3), the largest being (I/V)NS(N/D)GQ(M/L)(L/I).

p26

Polypeptide p26 is transcribed as early as 18 hours post infection (hpi) in the AcMNPV infection cycle (Bicknell *et al.*, 1987). The transcripts are synthesized by the host RNA polymerase II throughout the infection cycle (Huh and Weaver, 1990), accumulating in the cytoplasm. The peptide is normally present in the membrane fraction of budded virus (Goenka and Weaver, 1996) and has been shown to be non-essential for *in vitro* replication (Rodems and Friesen, 1993). AcMNPV *p26* gene produces two transcripts: a minor transcript terminating upstream of the p10 ORF and a major transcript transcribed through and co-terminating with the *p10* gene (Bicknell *et al.*, 1987). Unlike other baculoviruses sequenced to date, SeMPNV possesses two *p26* homologues. It would appear as though the two copies were acquired either independently from different sources, or that the one diverged from the other and then rearranged following duplication (Ijkel *et al.*, 1999). Although six *p26* baculovirus genes have been completely sequenced, the exact function has not been determined.

The TnSNPV putative *p26* gene, which appears to be the largest sequenced to date, is only the second SNPV *p26* gene identified. TnSNPV *p26* has previously been partially characterised (**Chapter 4** and Fielding and Davison, 2000). Amino acid sequence data was most homologous (36% identity and 53% similarity) to Se2MNPV p26 (Table 5.1). The ORF of 840 nts encodes for a possible protein 280 amino

Figure 5.4. Alignment of the deduced amino acid sequences of putative *p26* genes from six baculoviruses was done using the PileUp program at GCG. Dashes were introduced to align the sequences. Black shaded areas indicate 100% homology; deep grey 80% or more; light grey 60% or more. Se1MNPV (ORF81) and Se2MNPV (ORF128) were identified from one virus. Conserved motifs are over-lined. Refer to the Materials and Method section for baculoviruses used.

acids in length, with a putative mass of 31.71 kDa. Upstream of the p26 ORF a possible late promoter motif (ATAAG) is present at position -17nt relative to the putative ATG start codon. Further upstream, at position -230 nt and -251 nt, two characteristic early gene mRNA start sites (CAGT) were identified (van Oers *et al.*, 1998). However, these putative start sites were not preceded by perfect TATA boxes

Table 5.4. Identity and similarity (%) values of nine putative *p26* genes

	Se1MNPV	Se2MNPV	LdMNPV	AcMNPV	BmNPV	OpMNPV	CfMNPV	BuzuSNPV
TnSNPV	**13** 29	**36** 53	**20** 37	**22** 35	**22** 36	**18** 33	**19** 35	**34** 53
Se1MNPV		**19** 34	**18** 31	**15** 30	**14** 30	**13** 26	**15** 28	**19** 34
Se2MNPV			**27** 44	**28** 45	**26** 44	**26** 42	**26** 43	**45** 65
LdMNPV				**18** 34	**18** 33	**18** 32	**16** 31	**26** 45
AcMNPV					**93** 97	**44** 63	**52** 66	**30** 47
BmNPV						**41** 63	**50** 67	**29** 47
OpMNPV							**64** 78	**26** 42
CfMNPV								**25** 43

*values in bold indicate identity

as prescribed by van Strien *et al.* (1997). Downstream of the p26 ORF 2 possible poly-A motifs were present at position 41 nt and 51 nt relative to the putative stop codon TAA. The conserved p26 amino acid sequences - HQPPGV, GAPI, LVSVVT, SVYG, QLPY - are shown in Figure 5.4 (van Oers, 1998). However, it appears as though these amino acid sequences are less conserved than previously reported. Only two motifs seem to be highly conserved, *i.e.* (Y/F)PG and (I/V)S(V/L)(V/I)(T/S) (Figure 5.4).

SeORF-128 homologue

The function of the SeORF-128 homologue is not known. An ORF of 219 nucleotides encoding for a protein of 73 amino acids was identified, encoding for a putative protein of 8.48kDa. Two conserved

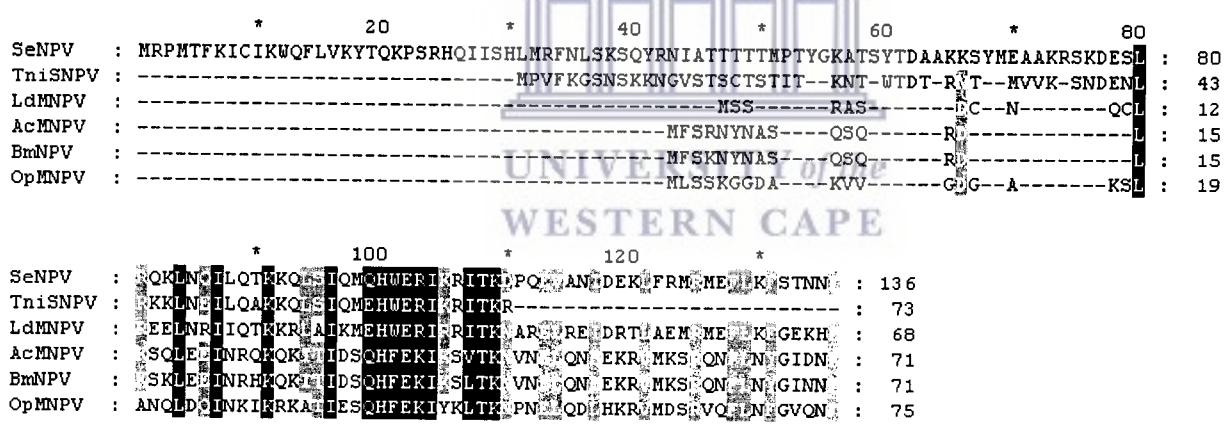


Figure 5.5. Alignment of the deduced amino acid sequences of SeORF-128 homologues using CLUSTAL X program. Dashes were introduced to align the sequences. Black shaded areas indicate 100% homology; deep grey 80% or more; light grey 60% or more. Refer to the Materials and Method section for baculoviruses used.

motifs were present (shaded in black): (Q/E)HW/FE(R/K)I and (I/L/V)TK (Figure 5.5). The TnSNPV ORF was similar in size to the ORFs of AcMNPV and OpMNPV. Comparison of TnSNPV to five homologues showed low identity values (Table 5.5). The SeORF-128 homologue primary amino acid sequence was not well conserved between the various NPVs. The TnSNPV SeORF-128 homologue was most homologues to SeMNPV with an identity value of 29% and a similarity value of 38%.

Table 5.5. Identity and similarity (%) values of SeORF-128 homologues

	SeMNPV	LdMNPV	AcMNPV	BmNPV	OpMNPV
TnSNPV	**29** 38	**21** 33	**14** 31	**15** 31	**12** 30
SeMNPV		**25** 38	**13** 32	**15** 32	**16** 33
LdMNPV			**25** 54	**25** 54	**26** 58
AcMNPV				**92** 100	**50** 69
BmNPV					**50** 69

for abbreviations see Materials and Methods section

p10

This non-structural peptide is expressed very late in the infection cycle. Although the gene is conserved between different baculoviruses, primary amino acid structures differ significantly. Particular segments of the gene confer unique functions on the virus (Wilson *et al.*, 1995). This suggests that the gene

evolved from an ancestor gene faster than other baculovirus genes. The TnSNPV *p10* gene has previously been identified on the *Eco*RI-V fragment and was subsequently characterised (**Chapter 4** and Fielding and Davison, 2000).

Arrangement of genes in the genome

Mechanisms involved in gene re-arrangement are not fully understood. However, it is known that the arrangement of genes of Group I NPVs is fairly conserved. On the other hand, the gene arrangement of

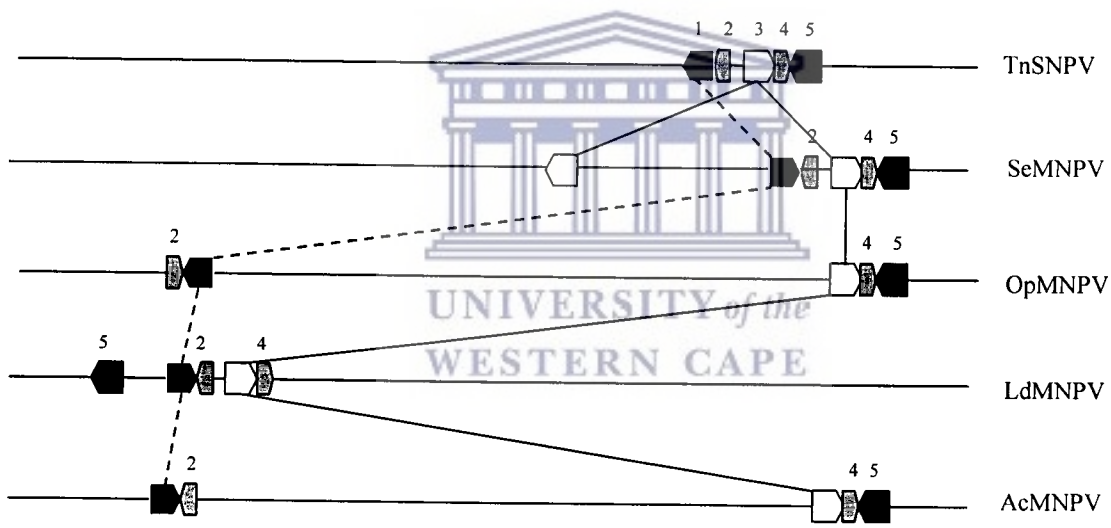


Figure 5.6: Comparison of the genomes of TnSNPV, AcMNPV, LdNPV, OpMNPV and SeNPV. Arrows represent the different ORFs and their respective directions of transcription (not drawn to scale). The *lef-6* (1) homologues are linked by dashed lines and the *p26* (3) homologues by solid lines. 2: SeORF-128; 4: *p10*; 5: *p74*

Group II NPVs is less conserved, indicating a more diverse group (Hu *et al.*, 1998). The cluster of genes identified (*lef*6-SeORF128-*p26-p10-p74*) was fairly well conserved between the different baculovirus genomes. With the exception of the transcriptional direction of the TnSNPV *lef*-6, the arrangement and direction of transcription of these genes in SeMNPV and TnSNPV were identical, indicating a possible common baculoviral ancestor. The entire LdMNPV gene cluster showed re-arrangement and a shift to the left of the genome. Gene arrangement and clustering of AcMNPV and OpMNPV are identical, with the gene duplex *lef*-6-SeORF128 shifted to the left (Figure 5.6). This confirmed the thought that LdMNPV is a distinct baculovirus with few relatives (Bulach *et al.*, 1999).

5.4.2 Phylogeny

In the past, genetic relationships between baculoviruses have been proposed by comparing host range, the degree of genomic hybridisation and by the degree of intertypic genomic recombination (Granados and Williams, 1986; Smith and Summers, 1982; Zanotto *et al.*, 1993). With the increase of available baculovirus sequence data, however, accurate estimations of phylogeny have become possible. Until recently, polyhedrin and granulin sequences were nearly exclusively used to determine the phylogenetic relationship between baculoviruses (Cowan *et al.*, 1994; Zanotto *et al.*, 1993). Phylogenetic studies based on the sequences of polyhedrin have shown that different lepidopteran NPVs have evolved from a common virus rather than from cross-infection from NPVs from other insect orders (Zanotto *et al.*, 1993). Furthermore, prior to the divergence of the distinct lepidopteran baculovirus NPV Groups I and II, a clade consisting of the GVs diverged early in baculovirus evolution (Zanotto *et al.*, 1993; Cowan *et al.*, 1994; Clarke *et al.*, 1996). Recently, however, the reliability of using occlusion body sequences as phylogenetic markers, have been questioned by van Strien *et al.* (1997). Since the

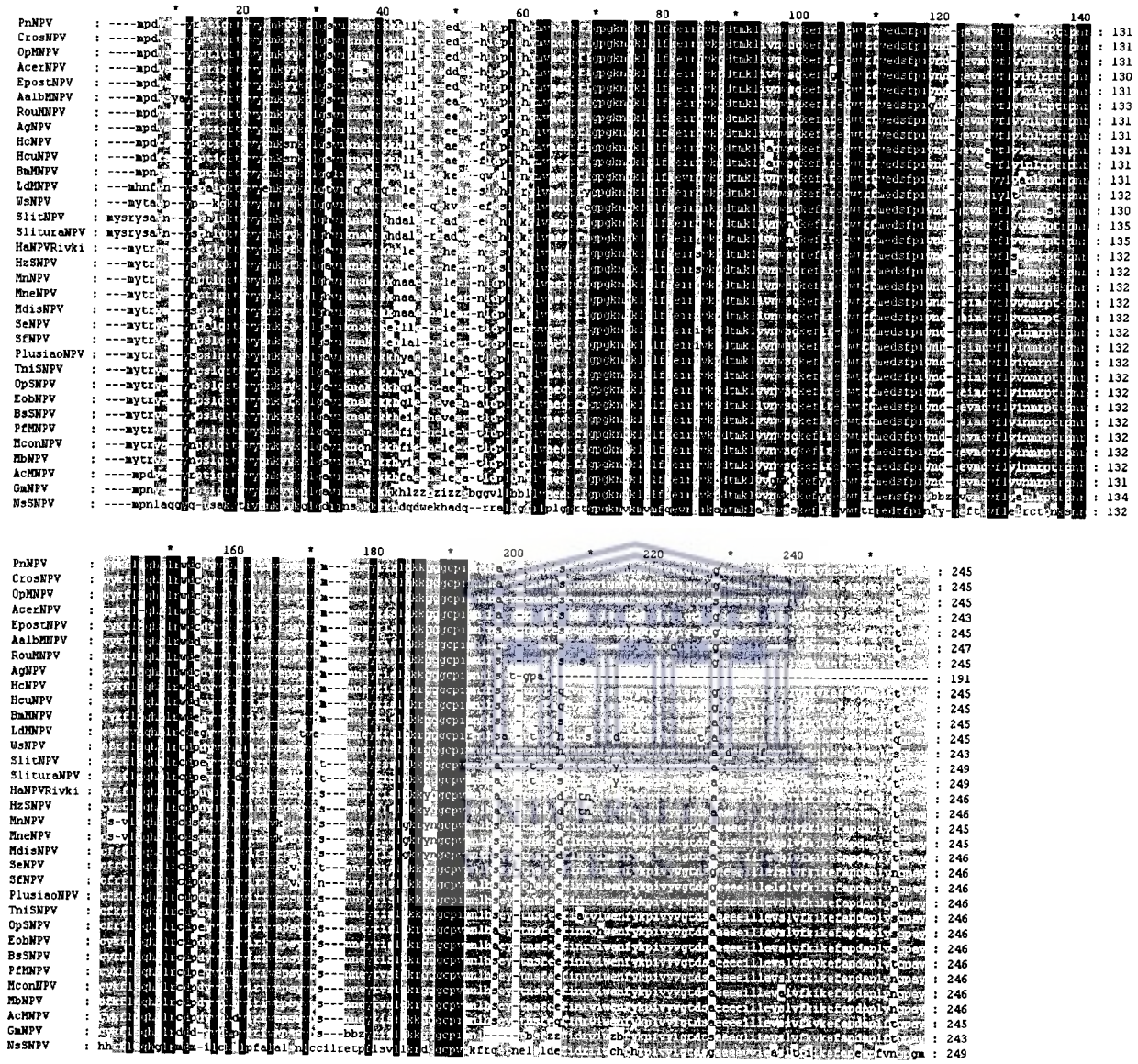


Figure 5.7. Alignment of the deduced amino acid sequences of 32 baculovirus polyhedrin genes was done using the PileUp program at GCG. Dashes were introduced to align the sequences. Black shaded areas indicate 100% homology; deep grey 80% or more; light grey 60% or more. Refer to the Materials and Method section for baculoviruses used.

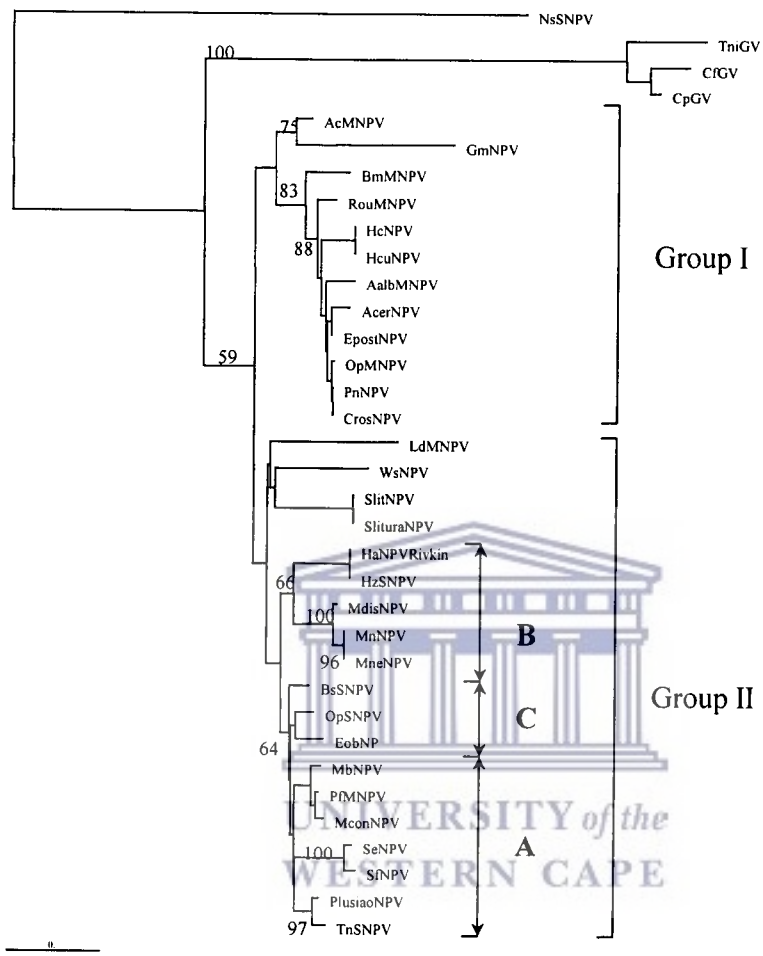


Figure 5.8. Evolutionary tree based on aligned polyhedrin amino acid sequences (Figure 5.6). The tree was estimated using the distance and neighbour-joining methods of CLUSTAL X. The tree is rooted using NsSNPV as outgroup. Selected bootstrap values following 100 replicates are indicated and branch lengths are scaled according to the number of expected steps. Group I and Group II (A, B and C) is shown.

protein is small in size (245 to 250 residues) and since more than half the residues are conserved, there are few informative sites to provide data for reliable phylogenetic analysis (Bulach *et al.*, 1999; van Strien *et al.*, 1997). Additionally, with occlusion body proteins there is no unambiguous outgroup criterion that can be used in phylogenetic estimations (Bulach *et al.*, 1999).

Therefore, trees should be constructed using more than one gene, so that differences between gene and organism trees can be identified (Clarke *et al.*, 1996; Bulach *et al.*, 1999). Although, no one particular inference procedure has emerged as a superior phylogenetic estimation tool (Nei, 1996), it would appear as though there is a statistical advantage of comparing trees under maximum likelihood. Furthermore, there is evidence that maximum likelihood is a superior tree-making algorithm (Bulach *et al.*, 1999). Ideally, though, several tree construction algorithms should be used for estimations.

Using gene arrangement and homology comparisons (**Chapter 2**), as well as the presence of conserved domains (**Chapter 3**), TnSNPV was previously classified as a putative Group II baculovirus. In this chapter different genes were used to confirm the phylogeny of TnSNPV. Consistent with previously reported trees (Zanotto *et al.*, 1993; Cowan *et al.*, 1994; Bulach *et al.*, 1999), there is consistent support for two distinct major clades in all estimated trees. In the occlusion body phylogenetic tree (Figure 5.7), three subclades appear to be present within Group II, called Subgroups II-A, -B and -C by Bulach *et al.* (1999). There is weak support (bootstrap value of 26) that LdMNPV, SlitNPV, SlituraNPV and WsNPV are Group II viruses, branching deep with no apparent close relatives. They are proposed to form a sister-group with the viruses included in Subgroups IIA-C. It appears as though the former diverged from the latter fairly soon after the divergence between Group I and Group II NPVs. Subgroup

II-A is proposed to consist of MbNPV, PfMNPV, MonNPV, SeNPV, SfNPV, the previously ungrouped PlusiaoNPV and TnSNPV. Subgroup II-B was proposed to consist of HaSNPV, HzSNPV MneNPV and the previously ungrouped MdisNPV and MnNPV. Finally, Subgroup II-C was proposed to contain BsSNPV, OpSNPV and EobNPV. This resolution of Subgroups IIA-C has been well supported by an earlier report. Trees generated from aligned data sets for *lef*-6, *p74* and *p26,* using a maximum likelihood algorithm, yielded trees with similar topologies to the tree generated from the polyhedrin sequences (results not shown).

Gene arrangement, gene homology and phylogenetic analysis confirmed the earlier speculation that TnSNPV was a Group II NPV (**Chapter 2** and **Chapter 3**). There is strong phylogenetic support that TnSNPV is member of Group II, specifically of Subgroup II-A. Furthermore, high bootstrap values indicated that SeNPV, SfNPV, PlusiaoNPV and TnSNPV share a recent common ancestor. This study confirmed earlier reports, that both gene arrangement and gene homology could be used as phylogenetic markers.

Summary

In South Africa there are more than 106 insects pests which attack a wide variety of crops. The top ten or twenty of these can seriously limit successful production on the farm. Costs involved in controlling these pests are considerable, often higher than the value of the crop itself. *Trichoplusia ni* (common name: cabbage looper) is a pest that can cause considerable damage to a wide variety of economically important crops. Although *Trichoplusia ni* has successfully been controlled with synthetic chemical pesticides, awareness about the negative impact of these control measures on the environment has necessitated the development of safer alternatives. Additionally, cabbage looper resistance to the commonly used pesticides has been reported.

Since a *Trichoplusia ni* multiple nuclear polyhedrosis virus has previously been used in the effective control of the pest, the potential of characterizing a South African baculovirus isolate showed great potential. A latent baculovirus infecting a field population of *Trichoplusia ni* was isolated and characterised. Initial DNA and protein characterization identified it as a novel baculovirus. The aim of this research was to characterise the baculovirus at a molecular level. This could lead to future improvement of the viral insecticidal properties.

The family Baculoviridae include more than 600 viruses with only nineteen receiving species status (Murphy *et al.*, 1995). The genome sequencing and mapping of NPVs could prove important in determining the relationships between these viruses. Additionally, it could be useful in understanding the importance of gene arrangement and the essential domains of

genes. This could provide insight into the *cis*- and *trans*-regulation among genes (Jin *et al.*, 1997). The determination of gene order and arrangement of the novel baculovirus isolated from a field population of *Trichoplusia ni* was presented in **Chapter 2**. Data were used to construct a partial functional map of the TniSNPV genome. Subsequently, the order and homology of genes identified were used as a phylogenetic marker, identifying TniSNPV as a putative member of the Group II NPVs.

Polyhedrin is the most conserved baculovirus protein sequenced and characterised thus far. It has been shown that polyhedrin is essential for the environmental persistence of the virus. Among lepidopteran baculovirus polyhedrin genes, amino acid identities are commonly in excess of 80%. Since the gene is not essential for infectivity or efficacy, foreign genes are often introduced *in lieu* of the coding region. Polyhedrin gene sequences have also been used to predict the phylogenetic relationship of baculoviruses (Zanotto *et al.*, 1993). The TniSNPV polyhedrin gene was sequenced (**Chapter 3**) as a prelude to the introduction of a foreign insect-specific toxin gene to increase the efficacy of the virus. The polyhedrin gene homologue identified in TniSNPV was most homologues to the polyhedrin gene from *Thysanoplusia oricalchea* MNPV. Finally, the gene sequence was used as additional speculative proof that TniSNPV is a Group II NPV.

Polypeptide p10 is non-structural protein that is expressed late in the infection cycle. Various regions within the gene have been shown to confer unique functions on the virus (Wilson *et al.*, 1995). Similarly to polyhedrin, the peptide is not essential for viral infectivity. Hence, the strong promoter is often used for expression of eukaryotic proteins in expression vector systems. Although *p10* has been conserved throughout baculovirus evolution, the primary peptide structure has not been conserved. This suggests that *p10* evolved from an ancestor

gene more rapidly than other baculovirus genes. The identification and characterization of the TniSNPV *p10* gene was described in **Chapter 4**. Even though the primary amino acid sequence of TniSNPV was not conserved, structural similarities to baculovirus homologues were identified. The gene was characterised as a prelude to using it in an eukaryotic expression vector system.

Comparative sequence analysis of specific regions of different baculoviruses has shown major differences in both gene content and arrangement (Theilmann and Steward, 1992). Although the relationships between gene arrangement and content of MNPV genomes have been studied extensively, much less is known about the relationships between SNPVs and MNPVs. Such comparisons could elucidate important information about baculovirus phylogeny. In **Chapter 5** a 2.97kb fragment of genome was sequenced and analysed. Analysis of such a large fragment could also reveal why the genome of TniSNPV is so much larger than that of the prototype virus AcMNPV. Based on sequence comparisons of various genes, lepidopteran NPVs have been classified into two phylogenetically distinct groups. A comprehensive knowledge of baculovirus phylogeny could be used to resolve the nomenclature of these viruses. Additionally, it could be useful in the future development of biological pesticides and eukaryotic expression systems based on intertypic recombinants (Zanotto *et al.*, 1993). Various TniSNPV genes were used to determine the phylogenetic relationship between TniSNPV and other baculoviruses. The TniSNPV gene arrangement in the genome was most similar to SeMNPV. TniSNPV was confirmed as a Group IIC NPV. As mentioned earlier, this knowledge could prove invaluable in future development of TniSNPV as either, an improved pesticide, or expression vector system.

Chemical pesticides have proven detrimental to both the environment and to human health. Therefore, safer, cheaper alternatives are constantly being sought. Baculoviruses have successfully been used to control various insect pests in different countries, particularly Brazil and China. A baculovirus isolated locally could prove ideal for the effective control of insect pests. However, before any attempt is made to genetically improve such a virus, a detailed study of the molecular dynamics involved in the virus need to be studied extensively. This thesis could present an important foundation for such future works.

APPENDIX

- **A sterilizable semi-synthetic diet for the rearing of lepidoptera larvae**

For 3.75 litres of diet the following was weighed:

Agar	75g
Casein	132g
Wheatgerm	288g
Wesson's salt	37.5g
Dried brewer's yeast	57g
Sucrose	117g
Cholesterol	3.75g
Methyl-4-hydroxy benzoate	3.75g
Sorbic acid	6.0g
Water	3450ml

The above ingredients were placed in a covered 5litre container and autoclaved for 20 minutes (at 20lb/sq inch). Following autoclaving, the ingredients were mixed thoroughly and cooled to 60^0C. Subsequently, the following was added:

Vitamin and antibiotic mixture	22.5g
Choline chloride	3.75g
Linseed oil	1.3ml

The mixture was poured into non-stick containers and allowed to solidify (van der Walt *et al.*, 1993).

- **Hybridisation techniques used**

The baked filter membrane was submerged in 10ml prehybridisation (5 x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent) solution in a hybridization tube and incubated for 3 hours at 65^0C in a hybridization oven. To denature the nick translated DNA probe, it was boiled at 95^0C for 10 minutes before being added to the hybridization solution. Following prehybridisation, the hybridisation solution (prehybridisation solution plus probe) was added to the tube and incubated for 16 hours at 65^0C. Subsequently, the filter was removed from the tube and washed twice in wash solution 1 (2xSSC, 0.1% SDS) for 15 minutes at room temperature. Also, the filter was washed twice in solution 2 (0.1x SSC, 0.1% SDS) for 15 minutes at 65^0C - about 200ml of each solution was used for each of the washes. Following the stringency washes, the filter was allowed to air dried before further use.

References

Adams, J.R. and J.T. McClintock. 1991. Baculoviridae, Nuclear polyhedrosis viruses, Part 1. Nuclear polyhedrosis viruses of insects. pp 89-147. *in* Adams, J.R. & Bonami, J.R. (Eds.) Atlas of Invertebrate pathology. United States of America, CRC Press Inc.

Adlerz, W. C. 1971. Cabbage looper control on watermelon at Leesburg, 1968-1970. Florida State Horticulture Society Proceedings: Annual Meeting. (8). 145-146.

Ahrens, C.H. and G.F Rohrman. 1995. Identification of essential *trans*-acting regions required for DNA replication of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus: *lef*-1 is an essential replication gene. Virology 207. 417-428.

Ahrens, C.H., Russel, R.L.Q., Funk, C.J., Evans, J.T., Harwood, S.H. and G.F. Rohrmann. 1997. The sequence of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. Virology 229. 381-399.

Akiyoshi, D.E., Chakerian, R., Rohrmann, G.F., Nesson, M.H. and G.S Bequdreau. 1985. Cloning and sequencing of the granulin gene from the *Trichoplusia ni* granulosis virus. Virology 141. 328-332.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and D.J Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programmes. Nucleic Acid Research 25, 3389-3402.

Arif, B.M. 1986. The structure of the viral genome. pp 21-29 *in* Doerfler, W. & Bohm, P. *(Eds.) The molecular biology of baculoviruses.* Berlin, Springer-Verlag.

Arnott, H.J. and K.M. Smith. 1968. Ultrastructure and formation of abnormal capsules in a granulosis virus of the moth *Plodia interpunctella* (Hübner). Journal of Ultrastructure Research 22.

136.

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

Bah, A., Bergeron, J., Arella, M., Lucarotti, C.J. and C. Guertin. 1997. Identification and sequence analyses of the granulin gene of *Choristoneura fumiferana* granulovirus, Archives of Virology 142: (8). 1577-1584.

Bansal, A., Bansal, O.B., Kumar, M., Behera, A.K. and R.H Das. 1997. Characterisation of the polyhedrin gene of *Spodoptera litura* nuclear polyhedrosis virus. Virus Genes. 14. (3). 175-180.

Barrett, J.W., Krell, P.J. and M.A. Basil. 1995. Characterization, sequencing and phylogeny of the ecdysteroid UDP-glucosyltransferase gene from two distinct nuclear polyhedrosis viruses isolated from *Choristoneura fumiferana*. Journal of General Virology 76. 2447-2456.

Bellett, A.J.D. 1969. Relationships among the polyhedrosis and granulosis viruses of insects. Virology 37. 117-123.

Berghold, G. 1947. Die isolierung des polyeder. Virus und die nature der polyeder. Naturforsch. 26. 122-143.

Bergold, G. H. 1953. On the nomenclature and classification of insect viruses. *Annual New York Academy of Science* 56. 595-616.

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.

Birnbaum, M.J., Clem, R.J. and L.K. Miller. 1994. An apotosis-inhibiting gene from the nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. Journal of Virology 68: (4). 2521-2528.

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

Bonning, B.C. and B.D. Hammock. 1996. Development of recombinant baculoviruses for insect control. Annual Review in Entomology 41. 191-210.

Bonning,, B.C. and B.D. Hammock. 1993. Lethal ratios: an optimised strategy for presentation of bioassay data generated from genetically engineered baculoviruses. Journal of Invertebrate Pathology 62. 196-197.

Braunagel, S.C. and M.D. Summers. 1994. Autographa californica nuclear polyhedrosis virus, PDV, and ECV viral envelopes andnucleocapsids: structural proteins, antigens, lipid and fatty acid profiles. Virology 202: (1). 315-28.

Bulach, D.M., Kumar, C.A., Zaia, A., Liang, B. and D.E. Tribe. 1999. Group II nucleopolyhedrovirus subgroups revealed by phylogenetic analysis of polyhedrin and DNA polymerase gene sequences. Journal of Invertebrate Pathology 73. 59-73.

Bump, N.J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., Li, P., Mankovrich, J., Shi, L., Greenberg, A.H., Miller, L.K. and W.W. Wong. 1995. Inhibition of ICE family proteases by baculovirus antiapoptotic protein *p35*. Science 269. 1885-1889.

Cameron, I.R. and R.D Possee. 1989. Conservation of polyhedrin gene promoter function between *Autographa californica* and *Mamestra brassicae* nuclear polyhedrosis viruses. Virus Research 125: (3922). 183-199.

Campbell, R.W. 1981. Population dynamics. *In*: The gypsy moth: research toward integrated pest management. C.C. Doane and M.L. Mcmanus (Eds.). USDA Technical Bulletin 1584.

Cartier, J.L., Hershberger P.A. and P.D. Friesen. 1994. Suppression of apoptosis in insect cells

stably transfected with baculovirus p35: dominant interference by N-terminal sequences p35 (1-76). Journal of Virology 68: (12). 7728-7737

Chakerian, R., Rohrmann, G.F., Nesson, M.H., Leisy, D.J. and G.S Beaudreau. 1985. The nucleotide sequence of the *Pieris brassicae* granulosis virus granulin gene Journal of General Virology 66: (6). 1263-1269

Chang, M.T., Lanner-Herrera, C. and M. Fikes. 1989. Nucleotide sequence of *Lymantria dispar* nuclear polyhedrosis virus polyhedrin gene. Journal of Invertebrate Pathology 53. 241-246.

Chen, X. and F. Carner. 1999. Characterization of a new multiple-nucleocapsid nuclear polyhedrosis virus from *Thysanoplusia orichalcea* (L) (Lepidoptera: Noctuidae), Accession number AF169480.

Chen, X., Hu, Z. and J.M Vlak. *Heliothis armigera* single-nucleocapsid nucleopolyhedrovirus polyhedrin gene. Accession number U97657.

Chen, X., Ijkel, F.J., Dominy, C., Zanotto, P.M.A., Hashimoto, Y., Faktor, O., Hayakawa, T., Wang, C-H., Prekumar, A., Mathavan, S., Krell, P.J., Hu, Z. and J.M. Vlak. 1999. Identification, sequence analysis and phylogeny of the *lef*-2 gene of Helicoverpa armigera single-nucleocapsid baculovirus. Virus Research. 65. 21-32.

Chen, X., Vlak, J.M. and Z.H. Hu. 1997. Nucleotide sequence analysis of the polyhedrin gene of *Heliothis armigera* single nucleocapsid nuclear polyhedrosis virus. Virology Sinica 12, 346-353.

Cheng X.W., Carner G.R. and H.W. Fescemyer. 1998. Polyhedrin sequence determines the tetrahedral shape of occlusion bodies in *Thysonaplusia oricalcea* single-nucleocapsid nucleopolyhedrosis virus. Journal of General Virology 79. 2549-2556.

Chou, C., Huang, C., Lo, C., Kou, G. and C. Wang. 1995. Detection, isolation and nucleotide sequences of *Perina nuda* multicapsid nuclear polyhedrosis virus (PnMNPV) polyhedrin gene.

Accession number U22824.

Clarke, E.E., Tristem, M., Cory, J.S. and D.R. O'Reilly. 1996. Characterization of the ecdysteroid UDP-glucosyltransferase gene of *Mamestra brassicae* nucleopolyhedrosisvirus. Jouranl of General Virology 77. 2865-2871.

Clem, R.J. and L.K. Miller. 1994. Control of programmed cell death by the baculovirus genes *p35* and *iap*. Molecular Cellular Biology 14. 5212-5222.

Clem, R.J., Fechheimer, M. and L.K. Miller. 1991. Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science 254: (5036). 1388-1390.

Cochran, M.A. and P. Faulkner. 1983. Location of homologous DNA sequences interspersed at five regions in the baculovirus AcMNPV genome. Journal of Virology 45; (3). 961-970.

Consigli, R.A., Tweeten, K.A., Anderson, D.K. and L.A. Bulla, Jr. 1983. Granulosis viruses, with the emphasis on the GV of the Indian meal moth, *Plodia interpunctella*. Advances in Virus Research. 28.141-173.

Cory, J.S., Hirst, M.L., Williams T., Hails R.S., Goulson D., Green B.M., Carty T.M., Possee R.D., Cayley P.J. and D.H.L. Bishop. 1994. Field trial of a genetically improved baculovirus insecticide. Nature 370. 138-140.

Cowan, P., Bulach, D., Goodge, K., Robertson, A. and D.E. Tribe. 1994. Nucleotide sequence of the polyhedrin gene region of *Helicoverpa zea* single nucleocapsid nuclear polyhedrosis virus: placement of the virus in lepidopteran nuclear polyhedrosis virus group II. Journal of General Virology 75. 3211-3218.

Crawford, A.M. and R.R. Granados. 1982. Nonoccluded baculoviruses. *In*: Procedures of Invertebrate Pathology Microbial Control. 3rd International Colloquium of Invertebrate Pathology. University of Sussex, Brighton, U.K. 154-159.

Croizier, L. and G. Croizier. 1994. Nucleotide sequence of the polyhedrin gene of *Spodoptera littoralis* multiple nucleocapsid nuclear polyhedrosis virus Biochem. Biophys. Acta 1218: (3). 457-459.

Crook, N.E. and J.D. Brown. 1982. Isolation and characterisation of a Granulosis virus from the tomatoe moth, *Lecanobia oleracea*, and its potential as a biological as a biological control agent. Journal of Invertebrate Pathology. 40. 221-227.

Crook, N.E., James, J.D., Smith, I.R.L. and D. Winstanley. Comprehensive physical map of *the Cydia pomonella* granulosis virus genome and sequence analysis of the granulin gene region. Unpublished.

Dai, X., Shi, X., Pang, Y. and D. Su. 1999. Prevention of baculovirus-induced apoptosis of BTI-Tn-5B1-4 (Hi5) cells by the p35 gene of *Trichoplusia ni* multicapsid nucleopolyhedrovirus Journal of General Virology 80: (7). 1841-1845.

Davis, T.R. and H.A. Wood. 1996. In vitro characterisation of a *Trichoplusia ni* single nucleocapsid nuclear polyhedrosis virus. Journal of General Virology. 77. 2303-2310.

Debolt, J.W., Wolf, W.W., Henneberry, T.J. and P.V. Vail. 1979. Evaluation of light traps and sex pheromone for control of cabbage looper and other lepidopterous insect pests of lettuce. USDA Technical Bulletin 1606.

Derksen, A.C.G. and R.R. Granados. 1988. Alteration of a Lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. Virology 167. 242-250.

Dornan, M.H., Sears, M.K. and J.G. Sears. 1995. Evaluation of a binomial model for insecticide application to control lepidopterous pests in cabbage. Journal of Economical Entomology 88. 302-306.

Du, Q., Lehavi, D., Faktor, Q., Qi, Y., and Y. Chejanovsky. 1999. Isolation of an apoptosis

suppressor gene of the *Spodoptera littoralis* nucleopolyhedrovirus. Journal of Virology 73; (2). 1278-1285.

Du, X. and S.M. Thiem. 1997. Characterisation of host range factor 1 (hrf-1) expression in *Lymantria dispar* M nucleopolyhedrovirus- and recombinant *Autographa californica* M nucleopolyhedrovirus-infected IPBL-Ld652Y cells. Virology 227. 420-430.

Duckett, C.S., Nava, V.E., Gedrich, R.W., Clem, R.J., van Dangen, J.L., Gilfillian, M.C., Shiels, H., Hardwick, J.M. and C.B. Thompson. 1996. A conserved family of cellular genes related to the baculovirus *iap* gene and encoding apoptosis inhibitors. The EMBO Journal 15. 2685-2694.

Dwyer, K.G. and R.R. Granados. 1987. A physical map of the *Pieris rapae* granulosis virus genome. Journal of General Virology 68. 1471-1476.

Efron, B. 1982. The Jackknife, the Bootsrap, and other resampling plans. CMBMS-NSF Regional Conference series in Applied Mathematics, Monograph 38. Society of Industrial and Applied Mathematics, Philadelphia.

Elkinton, J.S. and A.M. Liebhold. 1990. Population dynamics of gypsy moths in North America. Annual Review of Entomology 35. 571-596.

Erlandson, M.A., Keddie, B.A., Baldwin, D.J. and S. Li. A physical map *Malacosoma disstria* NPV genome and sequence analysis of the polyhedrin gene. Accession Number U61732.1.

Evans, H.F. and K.A. Harrap. 1982. Persistence of insect viruses. *In*: Virus persistence:Symposium 33. Society for general microbiology Ltd. Cambridge University Press.

Faktor, O., Toister-Achituv, M., Nahum, O., and B. Kamensky. 1997. The p10 gene of *Spodoptera littoralis* nucleopolyhedrovirus: nucleotide sequence, transcriptional analysis and unique gene organisation in the p10 locus. Journal of General Virology 78: (8). 2119-2128.

Faulkner, P., Kuzio, J., Williams, G.V. and J.A. Wilson. 1997. Analysis of p74, a PDV envelope protein of *Autographa californica* nucleopolyhedrovirus required for occlusion body infectivity *in vivo*. Journal of General Virology 78: (12). 3091-3100.

Faust, R.M. and J.R. Adams. 1966. The silicon content of nuclear and cytoplasmic virus inclusion bodies causing polyhedrosis in lepidoptera. Journal of Invertebrate Pathology. 8. 526-530.

Federici, B.A. and J.V. Maddox. 1996. Host specificity in microbe-insect interactions, Insect control by bacteria, fungal, and viral pathogens. Bioscience 46: (6). 410-421.

Federici, B.A. and R.H Hice. 1996. Organization and molecular characterization of genes in the polyhedrin region of the *Anagrapha falcifera* multinucleocapsid NPV. Accession number U64897.

Felsenstein, J. 1986. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39. 783-791.

Fielding B.C. and P.S. Davison. 2000. Identification and characterisation of the *Trichoplusia ni* single capsid nuclear polyhedrosis virus polyhedrin gene. Virus Genes 20: (2). 189-192.

Fielding, B.C. and P.S. Davison. 1999. The characterisation and phylogenetic relationship of the *Trichoplusia ni* single capsid nuclear polyhedrosis virus polyhedrin gene. Virus Genes 19: (1). 67-72.

Francki, R.I.B., Faquet, D.M., Knudsen, D.L. and F. Brown. 1991. Classification and nomenclature of viruses. *In* "The fifth report of the international committee on taxonomy of viruses". Archives of Virology. Supplement 2. 117-123.

Friesen, P.D. and L.K Miller. 1986. The regulation of baculovirus gene expression. *Current Topics in Microbiology and Immunology* 131. 31-49.

Friesen, P.D. and L.K. Miller. 1987. Divergent transcription of early 35- and 94-kilodalton protein

genes encoded by the *Hind*III K genome fragment of the baculovirus *Autographa californica* nuclear polyhedrosis virus. Journal of Virology 617. 2264-2272.

Fuchs, L.Y., Woods, M.S. and R.F. Weaver. 1983. Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection: a novel RNA polmerase induced in infected *Spodoptera frugiperda* cells. Journal of Virology 43. 641-646.

Fuxa, J.R., Weidner, E.H. and A.R. Richter. 1992. Polyhedra without virions in a vertically transmitted nuclear polyhedrosis virus. Journal of Invertebrate Pathology 60. 53-58.

Gish, W. and D. J. States. 1993. Identification of protein coding regions by database similarity search. National Genetics 3. 266-72.

Goenka, S. and R.F. Weaver. 1996. Cellular localization of p26, an early AcMNPV gene product. *In:* Abstract of the 15th Annual meeting of the American Society of Virology. p154.

Gombart, A.F., Blissard, G.W. and G.F. Rohrmann. 1989. Characterisation of the *Hind*III M region of the multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata* reveals major differences among baculoviruses. Journal of General Virology 80. 1815-1828.

Gombart, A.F., Pearson, M.N., Rorhmann, G.F. and G.S. Beaudreau. 1989. A baculovirus polyhedral envelope-associated protein: Genetic location, nucleotide sequence, and Immunocytochemical characterisation. Virology 169. 182-193.

Gomi, S., Majima, K. and S. Maeda. 1999. Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. Journal of General Virology 80. 1323-1337

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

Goto, C. and S. Maeda. Genome organization of *Xestia c-nigrum* granulosis virus, Unpublished.

Granados, R.R. 1978. Early events in the infection of *Heliothis zea* mid-gut cells by a baculovirus. Virology 90. 170.

Granados, R.R. and K.A. Williams. 1986. In vivo infection and replication of Baculviruses. *In*: The biology of Baculoviruses. R.R. Granados, B.A. Frederici (Eds.). Boca Raton, Florida: CRC.

Hammock B.D., Bonning B.C., Possee R.D., Hanzlik T.N. and S. Maeda. 1990. Expession and effects of the juvenile hormone esterase in a baculovirus system. Nature 344. 458-461.

Hardin, S.E. and R.F. Weaver. 1990. Overlapping divergent transcripts mapping to the *Hind*III F region of the *Autographa californica* nuclear polyhedrosis virus. Journal of General Virology 71. 225-229.

Harrison, R.L. and B.C Bonning. 1999. The nucleopolyhedroviruses of *Rachiplusia ou* and *Anagrapha falcifera* are isolates of the same virus. Journal of General Virology 80: (10). 2793-2798.

Hayakawa, T., Ko, R., Okano, K., Seong, S.I., Goto, C. and S. Maeda. 1999. Sequence analysis of the *Xestia c-nigrum* granulovirus genome. Virology 262: (2). 277-297.

Hershberger, P.A., Dickson, J.A. and P.D. Friesen. 1992. Site-specific mutagenesis of the 35-kilodalton protein gene encoded by *Autographa californica* nuclear polyhedrosis virus: cell line-specific effects on virus replication. Journal of Virology 66. 5525-5533.

Hershberger, P.A., LaCount D.J. and P.D. Friesen. 1994. The apoptotic suppressor P35 is required early during baculovirus replication and is targeted to the cytosol of infected cells. Journal of Virology 68: (6). 3467-3477.

Higgins, D.G. and P.M. Sharpe. 1988. CLUSTAL W: a package for performing multiple sequence alignment on a microcomputer. Gene 73. 237-244.

Hill, J.E., Kuzio, J., Wilson, J.A., MacKinnon, E.A. and P. Faulner. 1993. Nucleotide sequence of the *p74* gene of a baculovirus pathogenic to the spruce budworm, *Choristineura fumifera*na multicapsid nuclear polyhedrosis virus. Biochimica Biophysica Acta 1172. 187-189.

Hoffman, M.P. and A.C Frodsham. 1993. Natural enemies of vegetable insect pests. New York, Cornell Cooperative Extension Publication. p 5.

Hong, T., Braunagel, S.C. and M.D. Summers. 1994. Transcription, translation and cellular localisation of PDV-E66: A structural protein of the PDV envelope of *Autographa californica* nuclear polyhedrosis virus. Virology 204. 210-209.

Hooft Van Iddekinge, B.J.L., Smith, G.E. and M.D. Summers. 1983. Nucleotide sequence of the polyhedrin gene of *Autographa californica* nuclear polyhedrosis virus. Virology 131. 561-565.

Howard, C.R. 1986. The biology of hepadnaviruses. Journal of General Virology 67. 1215-1235.

Hu, Z.H., Arif, B.M., Jin, F., Martins, J.W.M., Chen, X.W., Sun, J.S., Zuidema, D., Goldbach, R.W. and J.M. Vlak. 1998. Genetic organisation of the *Hind*III-I region of the single-nucleocapsid nucleopolyhedrovirus of *Buzura supressaria*. Virus Research 55. 71-82.

Hu, Z.H., Broer, R., Westerlaken, J., Martens, J.W., Jin, F., Jehle, J.A., Wang, L.M. and J.M. Vlak. 1997. Characterisation of the ecdysteroid UDP-glucosyltransferase gene of a single nucleocapsid nucleopolyhedrovirus of *Buzura suppressaria*. Virus Research 47. 91-97.

Hu, Z.H., Liu, M.F., Jin, F., Wang, Z.X., Liu, X.Y., Li, M.J., Liang, B.F. and T.E. Xie. 1993. Nucleotide sequence of the *Buzura suppressaria* single nucleocapsid nuclear polyhedrosis virus polyhedrin gene. Journal of General Virology 74: (8). 1617-1620.

Hughes D.S., Possee R.D. and L.A. King. 1993. Activation and detection of a latent baculovirus resembling *Mamestra brassicae* nuclear polyhedrosis virus in *M. brassicae* insects. Virology 194. 608-615.

Hughes, D.S., Possee, R.D. and L.A. King. 1997. Evidence for the presence of a low-level, persistent baculovirus infection of *Mametra brassicae* insects. Journal of General Virology 78. 1801-1805.

Hughes, P.R. and H.A. Wood. 1981. A synchronous peroral technique for the bioassay of insect viruses. Journal of Invertebrate Pathology. 37. 154-159.

Huh, N.E. and R.F. Weaver. 1990. Identifying the RNA polymerases that synthesize specific transcripts of *Autographa californica* nuclear polyhedrosis virus. Journal of General Virology 71. 195-202.

Hyink, O., Graves, S., Fairbairn, F.M. and V.K Ward. 1998. Mapping and Polyhedrin Gene Analysis of the *Epiphyas postvittana* nucleopolyhedrovirus genome. Journal of General Virology 78: (11). 2853-2862.

Iatrou, K., Ita, K. and H. Witkiewicz. 1985. Polyhedrin gene of *Bombyx mori* nuclear polyhedrosis virus. Journal of Virology 54. 436-445.

Ignofo, C.M. 1979. Viral bioinsecticides. In J.R. Porter (Ed.), Under-exploited microbial processes of potential economic importance. National Academy of Science. Washington, D.C. 142-153.

Ijkel, W.F.J., van Strien, E.A., Heldens, J.G.M., Broer, R., Zuidema, D., Goldbach, R.W. and J.M. Vlak. 1999. Sequence and organisation of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. Journal of General Virology 80. 3289-3304.

Isayama, S., Yasuhisa, K. and K. Yasui. Nucleotide sequence of *Hyphantria cunea* nuclear polyhedrosis virus polyhedrin gene. Accession number D14573.

Jankevica, L., Kropa, M. and Jankevics, E. Comparision of isolates of *Malacosoma neustria* nucleopolyhedroviruses by sequencing of PCR products. Accession number X55658.

Jehle, J.A. and H. Backhaus. 1994. The granulin gene region of *Cryptophelia leucotreta* granulosis

virus: sequence analysis and phylogenetic considerations. Journal of General Virology 75. 3667-3671.

Jehle, J.A., van der Linde, I.F.A., Backhaus, H. and J.M. Vlak. 1997. Identification and sequence analysis of the integration site of transposon TCp3.2 in the genome *of Cydia pomo*nella granulovirus. Virus Research. 50. 151-157.

Jin, T., Qi, Y., Liu, D. and F. Su. 1999. Nucleotide sequence of a 5892 base pairs fragment of the LsMNPV genome and phylogenetic analysis of LsMNPV. Virus Genes 18: (3). 265-76.

Jin, T., Qi, Y., Qi, B., Jin, H. and Y. Huang. 1997. Nucleotide sequence of a 5423 base pairs fragment of the LsNPV genome and comparison with the AcNPV genome. Virus Genes 14: (3). 201-210.

Kamita, S.G., Majima, K. and S. Maeda. 1993. Identification and characterisation of the p35 gene of *Bombyx mori* nuclear polyhedrosis virus that prevents virus-induced apoptosis. Journal of Virology 67: (1). 445-463.

Kang, W., Crook, N.E., Winstanley, D. and D.R. O'Reilly. 1997.Complete sequence and transposon mutagenesis of the *Bam*HI-J fragment of *Cydia pomonella* Granulosis virus. Virus Genes 14: (2). 131-136.

Ke, A., Gillock, E.T., Sweat, J.A., Reeves, W.M. and R.A. Consigli. 1999. Use of the baculovirus system to assemble polyomavirus capsid-like particles with different polyomavirus structural proteins: analysis of the recombinant assembled capsid-like particles. Journal of General Virology 80. 1009-1016.

Kelly, D.C. 1982. Review article: Baculovirus replication. Journal of General Virology 63. 1-13.

Kelly, D.C. and T. Lescott. 1981. Baculovirus replication: protein synthesis in *Spodoptera frugiperda* cells infected with *Trichoplusia ni* nuclear polyhedrosis virus. Microbiologica. 4. 35-47.

Kelly, D.C., Lescott, T., Ayres, M.D., Carey, D., Coutts, A. and K.A. Harrap. 1981. Induction of a nonoccluded baculovirus persistently infecting *Heliothis zea* cells by *Heliothis armigera* and *Trichoplusia ni* nuclear polyhedrosis viruses. Virology 112. 174-189.

King L.A. and R.D. Possee. 1994. Anvances in Insect Virology. *In*: Advances In Insect Physiology, Volume 25. E. Evans (Ed.). Academic Press. Harcourt Brace and Company, Publishers. London. 2-29.

King, L.A. and R.D. Possee. 1992. The baculovirus expression system: a laboratory guide. Chapman Hall, London.

Kishaba, A.N., Whitaker, T.W., Berry, W. and H.H. Toba. 1976. Cabbage Looper Oviposition and Survival of Progeny on Leafy Vegetables. Hortscience 11: (3). 216-217.

Kondo, A. and S. Maeda. 1991. Host range expansion by recombination of the baculoviruses *Bombyx mori* nuclear polyhedrosis virus and *Autographa californica* nuclear polyhedrosis virus. Journal of Virology 65: (7). 3652-3632.

Kool, M. and J.M. Vlak. 1993. The structural and functional organization of the *Autographa californica* nuclear polyhedrosis virus genome- Review. Archives of Virology 130: (1-2). 1-16.

Kool, M., Ahrens, C.H, Golgbach, R.W., Rorhmann, G.F. and J.M. Vlak. 1995. Identification of genes involved in DNA replication of the *Autographa californica* baculovirus. Proceedings of the National Academy of Science, USA 91. 11212-11216.

Kool, M., Ahrens, C.H., Goldbach, R.W., Rohrmann, G.F. and J.M. Vlak. 1994. Identification of genes involved in DNA replication of the *Autographa californica* baculovirus. Proceedings of the National Academy of Science U.S.A 91. 11212-11216.

Kool, M., Ahrens, C.H., Vlak, J.M. and G.F. Rorhmann. 1995. Review article: Replication of baculovirus DNA. Journal of Virology (?). 2103-2118.

Kost, T.A. and J.P. Condreay. 1999. Recombinant baculoviruses as expression vectors for insect and mammalian cells- Review. Current Opinion in Biotechnology 10: (5). 428-33.

Kozlov, E.A., Rodnin, N.V., Levitina, T.L., Gusak, N.M., Radomskij, N.F. and L.J. Palchikovskaya. 1992. The amino acid sequence determination of a granulin and polyhedrin from two baculoviruses infecting *Agrotis segetum*. Virology 189: (1). 320-323.

Kropa, M. and E. Jankevics. Comparision of isolates of *Malacosoma neustria* nucleopolyhedroviruses by sequencing of PCR products. Unpublished.

Kukan B. 1999. Vertical transmission of nucleopolyhedrovirus in insects- Review. Journal of Invertebrate Pathology 74: (2). 103-111.

Kunimi, Y., Fuxa, J.R. and B.D. Hammock. 1996. Comparison of wild type and genetically engineered nuclear polyhedrosis virusesof *Autographa californica* for mortality, virus replication and polyhedra production in *Trichoplusia ni* larvae. Entomologia Experimentalis *et* Applicata 81. 251-257.

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

Kuzio, J., Pearson, M.N. Harwood, S.H., Funk, C.J., Evans, J.T., Slavicek, J.M. and G.F. Rohrmann. 1999. Sequence and analysis of the genome of a baculovirus pathogenic for *Lymantria dispar*. Virology 253: (1). 17-34.

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

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

Lacount, D.J. and P.L. Friesen. 1997. Role of early and late replication events in induction of apoptosis by baculoviruses. Journal of Virology 71. 1530-1537.

Lee, H.H., Chung, H.K. and N-S. Seo. 1992. Genomic structure and nucleotide sequence of the polyhedrin gene of *Hyphantria cunea* nuclear polyhedrosis virus. Mol. Cells 2. 303-308.

Leisy, D.J., Nesson, M., Pearson, M., Rohrmann, G.F. and G.S Beaudreau. 1986a. Location and nucleotide sequence of the *Orgyia pseudotsugata* single nucleocapsid nuclear polyhedrosis virus polyhedrin gene. Journal of General Virology 67. 1073-1079.

Leisy, D.J., Nesson, M., Pearson, M., Rohrmann, G. and G.S. Beaudreau 1986b. Nucleotide sequencing and transcriptional mapping of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus p10 gene. Virology 153. 157-167.

Leisy, D.J., Rohrmann, G. and G. Beaudreau. 1986. The nucleotide sequence of the polyhedrin gene region from the multicapsid baculovirus of *Orgyia pseudotsugata*. Virology 153: (2). 280-288.

Li, S., Erlandson, M., Moody, D. and C. Gillott. 1997. A physical map of the *Mamestra configurata* nucleopolyhedrovirus genome and sequence analysis of the polyhedrin gene. Journal of General Virology 78: (1). 265-271.

Liu, J.C. and J.E. Maruniak. 1999. Molecular characterization of genes in the GP41 region of baculoviruses and phylogenetic analysis based upon GP41 and polyhedrin genes. Virus Research 64: (2). 187-196.

Longworth, J.F. and J.C. Cunningham. 1968. The activation of occult nuclear-polyhedrosis viruses by foreign nuclear polyhedra. Journal of Invertebrate Pathology 10. 361-367.

Lu, A., and L.K. Miller. 1995. The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication. Journal of Virology 69: (2). 975-982.

Lu, A., Kriel, P.J., Vlak, J.M. and G.F. Rohrmann. 1997. Baculovirus DNA replication. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum Press. New York. Pp 171-191.

Lucarotti, C.J. and B. Morin. 1997. A nuclear polyhedrosis virus from the oblique banded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae). Accession number U91940.

Maeda, S. 1995. Further developments of recombinant baculovirus insecticides. Current Opinion in Biotechnology 6. 313-319.

Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., Saeki, Y., Sato, Y., and M. Furusawa. 1985. Production of human α-interferon in silkworm using a baculovirus vector. Nature 315: (13). 592-594.

Marsden, D. A. 1979. Insect Pest Series No. 6 Cabbage Worm. Cooperative Extension Service, University of Hawaii, College of Tropical Agriculture and Human Resources.

Martignoni, M.E. and P.J. Iwai. 1986. A catalogue of viral diseases of insects, mites and ticks. U.S. Department of Agriculture, Forestry Services Pacific Northwest, Research Station General Technical Rep. PNW-195. 4th edition revised. p50.

Matthews, R.E.F. 1982. Classification and nomenclature of viruses. Intervirology 17, 1-200.

McKinley, D.J., Brown, D.A., Payne, C.C. and K.A. Harrap. 1981. Cross-infectivity and activation studies with four baculoviruses. Entomophaga 26. 79-90.

McLachlin, J.R. and L.K. Miller. 1994. Identification and characterisation of *vlf*-1, a baculovirus gene involved in very late gene expression. Journal of Virology 68. 7746-7756.

Mellerik, D.M. and N.W. Fraser. 1987. Physical state of the latent herpes simplex virus genome in a mouse model system: Evidence supporting an episomal state. Virology 158. 265-275.

Merrington, C.L., Kitts, P.A., King, L.A. and R.D. Possee. 1996. An *Autographa californica* nucleopolyhedrovirus *lef*-2 mutant: consequences for DNA replication and very late gene expression. Virology. 217. 338-348.

Merryweather A.T., Weyer U., Harris M.P.G., Hirst M., Booth T. and R.D. Possee. 1990. Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. kurstaki HD-73 delta toxin. Journal of General Virology 71. 1535-1544.

Miller LK. 1997. Baculovirus interaction with host apoptotic pathways. Journal of Cell Physiology 73: (2). 178-182.

Miller, L.K. 1988. Baculovirus as gene expression vectors. Annual Review Microbiology 42. 177-199.

Miller, L.K. 1993. Current Opinion in Genetic Development. 97-101.

Miller, L.K. 1995. "1994 Founders Lecture". Genetically engineered insect virus pesticides: Present and Future. Journal of Invertebrate Pathology 65. 211-216.

Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A. and M.D Summers. (Eds.). 1995. Virus taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses.

Nei, M. 1996. Phylogenetic analysis in molecular evolution. Annual Review in Genetics 30. 371-403.

Newcomb, W.W., Homa, F.L., Thomsen, D.R., Booy, F.P., Trus, B.L., Steven, A.C., Spencer, J.V. and J.C. Brown. 1996. Assembly of the herpes simplex virus capsid: characterisation of intermediates observed during cell-free capsid assembly. Journal of Molecular Biology 263. 432-446.

Nicholas, K.B. and H.B Nicholas Jr. 1997. GeneDoc: a tool for editing and annotating multiple sequence alignments. *Distributed by author.*

O'Reilly, D.R. 1997. Auxiliary genes of baculoviruses. *In*: The baculoviruses. L.K. Miller (Ed.). Plenum Press, New York. 267-300.

O'Reilly, D.R. and L.K. Miller. 1991. Improvement of a baculovirus pesticide by deletion of the *egt* gene. Bio-Technology 9. 1086-1089.

Oakey, R., Cameron, I.R., Davis, B., Davis, E. and R.D. Possee. 1989. Analysis of transcription initiation in the *Panolis flammea* nuclear polyhedrosis virus polyhedrin gene. Journal of General Virology 70: (3). 769-775.

Oellig, C., Happ, B., Muller, T. and W. Doerfler. 1987. Overlapping sets of viral RNAs reflect the array of polypeptides in the *Eco*RI J and N fragments (map position 81.2 to 85.0) of the *Autographa californica* nuclear polyhedrosis virus. Journal of Virology 61. 3048-3057.

Page, R.D.M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12. 357-358.

Pari, G.S., Kacica, M.A. and D.A. Anders. 1993. Open reading frames UL44, IRS1/TRS1, and UL36-38 are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA synthesis. Journal of Virology 67. 2575-2582.

Passarelli, A.L. and L.K. Miller. 1994. Identification and transcriptional regulation of the baculovirus *lef*-6 gene. Journal of Virology 68: (7). 4458-4467.

Pennock, G.D., Shoemaker, C. and L.K. Miller. 1984. Strong and regulated expression of *Eschericia coli* β-galactosidase in insect cells with a baculovirus vector. Molecular and Cellular Biology. 4. 399-406.

Pikhod'ko, E.A. and L.K. Miller. 1999. The baculovirus PE38 protein augments apoptosis induced by transactivator IE1. Journal of Virology 73: (8). 6691-6699.

Pingel, R.L. and L.C. Lewis. 1997. Field application of *Bacillus thuringiensis* and *Anagrapha falcifera* multiple nucleopolyhedrovirus against the corn earworm. (Leidoptera: Noctuidae). Journal of Economic Entomology 90: (5). 1195-1199.

Ponsen, M.B. and D.J. De Jong. 1964. On a nuclear and cytoplasmic polyhedrosis of *Adoxophyes reticulana* HB. Entomophaga 9: (3). 253-255.

Possee, R.D. 1986. Cell-surface expression of influenza virus haemagglutonin in insect cells using a baculovirus vector. Virus Research 5. 43-59.

Premkumar, A. and S. Mathavan. Polyhedrin gene sequence of *Amsacta albistriga* nuclear polyhedrosis virus (AaMNPV). Accession number AF118850.

Prikhod'ko, E.A. and L.K. Miller. 1996. Induction of Apoptosis by Baculovirus transactivator *IE1*. Journal of Virology 70. 7116-7124.

Prikod'ko, G.G., Robson, M., Warmke, J.W., Cohen, C.J., Smith, M.M., Wang, P., Warren, V., Kaczorowski, G. van der Ploeg L.H.T. and L.K. Miller. 1996. Properties of three baculovirus-expressing genes that encode insect-selective toxins: μ-Aga-IV, AsII and ShI. Biological Control 7. 236-244.

Qin, J.C., Liu, A.F. and R.F. Weaver. 1989. Studies on the control region of the p10 gene of *the Autographa californica* nuclear polyhedrosis virus. Journal of General Virology 70: (5). 1273-1279.

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

Rieth,A., Valero, J.R., Letarte, R., Bourque, S.N. and R.M. Tanguay. Identification of the

polyhedrin gene of three baculovirus pathogenic to the spruce budworm, *Choristoneura fumiferana.* accession U40834.1

Rivkin, H., Mor, M. and N. Chejanovsky. 1998. Isolation, replication and polyhedrin gene sequence of an Israeli *Helicoverpa armigera* single nucleopolyhedrosis. Virus Genes 17: (1). 11-19.

Rodems, S.M and P.D. Friesen. 1993. The *hr*5 transcriptional enhancer stimulates early expression from the *Autographa californica* nuclear polyhedrosis virus genome but is not required for virus replication. Journal of Virology 67. 5776-5785.

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

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

Sadler, T.J., Glare, T., Ward, V.K. and J. Kalmakoff. The polyhedrin gene from a nucleopolyhedrovirus of *Wiseana signata* (Lepidoptera: Hepialidae). Accession number AF016916.

Saitou, N. and M. Nei. 1987. The neighbour joining method: A new method for reconstructing phylogenetic trees. Molecular and Biological Evolution 4. 406-425.

Sambrook, J., Fritsch, E.F. and T. Maniatis. 1989. Molecular cloning, A laboratory manual, 2nd edition number 1, 2, 3. United States of America, Cold Spring Harbour, Cold Spring Harbour Press.
Schaffer, H.E. and R.R. Sederhoff. 1981. DNAFRAG, version 3.03. Analytical Biochemistry 115, 113-122.

Slack, J.M. and G.W. Blissard. 1997. Identification of two independent transcriptional activation domains in the *Autographa californica* multicapsid nuclear polyhedrosis virus IE1 protein. Journal of Virology 71: (12). 9579-9587.

Smirnoff, W.A. 1965. Observations on the effect of virus infection on insect behaviour. Journal of Invertebrate Pathology 7. 387-391.

Smith, D.H., da Graca and V.H. Whitlock. 1990. Granulosis virus from *Cacoecia occidentalis*: Isolation and morphological description of a Granulosis virus of the citrus leafroller, *Cacoecia occidentalis*. Journal of Invertebrate Pathology 55. 319-324.

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

Smith, G.E. and M.D. Summers. 1982. DNA homology among subgroups A, B and C baculoviruses. Virology. 123. 399-406.

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

Smith, G.E., Ju, G., Ericson, B.L., Maschera, J., Lahm, H-W., Chizzonite, R. and M.D. Summers. 1985. Modification and secretion of human interleukin 2 produced in insect cells by a baculovirus expression vector. Proceedings of the National Academy of Science USA 82. 8404-8408.

Smith, I. and C. Goodale. 1998. Sequence and in vivo transcription of *Lacanobia oleracea* granulovirus *egt*. Journal of General Virology. 79. 405-413.

Smith, I.R.L. and N.E. Crook. 1988. Physical maps of the genomes of four variants of *Artogeia rapae* granulosis virus. Journal of General Virology 69. 1741-1747.

Smith, I.R.L., van Beek, N.A.M., Podgwaite, J.D. and W.A Wood. 1988. Physical map and polyhedrin gene sequence of *Lymantria dispar* nuclear polyhedrosis virus. Gene 71. 97-105.

Smith, K.M. 1963. The cytoplasmic virus diseases. *In* E.A. Steinhaus (Ed.): Insect pathology: An advanced treatise (volume 1). 457-497. Academic Press. New York.

Stairs, G.R., Parrish, W.B., Briggs, T.D. and M. Allietta. 1966. Fine structure and of a granulosis

virus of codling moth. Virology. 30. 583-584.

Steller, H. 1995. Mechanisms and genes of cellular suicide. Science 267, 1445-1449.

Stewart, L.M.D., Hirst, M., Ferber, M.L., Merryweather, A.T., Cayley, P.J. and R.D. Possee. 1991 Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. Nature 352. 85-88.

Theilmann, D.A. and S. Steward. 1992. Molecular analysis of the trans-activating IE-2 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. Virology 187. 84-96.

Thiem, S.M., Du, X., Quentin, M.E. and M.E. Merner. 1996. Identification of a baculovirus gene that promotes *Autographa californica* nuclear polyhedrosis virus replication in a nonpermissive cell line. Journal of Virology 70: (4). 2221-2229.

Thompson, J.D, Higgins D.G. and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22. 4673-4680.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and D.G Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24. 4876-4882.

Tinsley, T.W. and D.C. Kelly. 1985. Taxonomy and nomenclature of insect pathogenic viruses. *In*: Viral insecticides for biological control. K. Maramorosch and K.E. Sherman (Eds.). Academic Press, Orlando, Florida.

Tomalski M.D. and L.K. Miller. 1991. Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. Nature 352. 82-85.

Tweeten, K.A., Bulla, Jr. L.A. and R.A. Consigli. 1981. Applied and Molecular aspects of insect

granulosis viruses. Microbiological Reviews 45. 379-408.

Uren, A.G., M. Pakusch, C.J. Hawkins, K.L. Puls and D.L. Vaux. 1996. Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. Proceedings of the National Academy of Science USA 93. 4974-4978.

Van der Walt , Botes, S.J., Basson R.I. and J.D. Mohr. 1993. Rearing *Heliothis armigera* (*Lepidoptera*: Noctuidae) in the laboratory and criteria to distinguish the larval instars. Phytophylactica 25. (3): 65-66.

Van der Wilk, F., van Lent, J.W.M. and J.M. Vlak. 1987. Immunogold detection of polyhedrin, p10 and virion antigens in *Autographa californica* nuclear polyhedrosis virus-infected *Spodoptera frugiperda* cells. Journal of General Virology 69. 2615-2623.

Van Oers M.M., Flipsen J.T.M., Reusken C.B.E.M., and J.M. Vlak. 1994. Specificity of baculovirus *p10* functions. Virology 200 513-523.

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

Van Oers M.M., Hu Z., Arif B.M., van Strien E.A., van Lent J.W.M., and J.M Vlak. 1998. The single-nucleocapsid nucleopolyhedrovirus of *Buzura suppressaria* encodes a p10 protein. Journal General Virology 79. 1553-1562.

Van Oers, M.M. and J.M. Vlak. 1997. The baculovirus 10-kDa protein. Journal of Invertebrate Pathology 70: (1). 1-17.

van Strien, E.A., Faktor, O., Hu, Z.H., Zuidema, D., Goldbach, R.W. and J.M. Vlak. 1997. Bacolviruses contain a gene for the large subunit of ribonucleotide reductase. Journal of General

Virology 78. 2365-2377.

van Strien, E.A., Zuidema, D., Goldbach, R.W. and J.M. Vlak. 1992. Nucleotide sequence and transcriptional analysis of the polyhedrin gene of *Spodoptera exigua* nuclear polyhedrosis virus. Journal of General Virology 73: (11). 2813-2821.

Vlak, J.M. and G.E. Smith. 1982. Orientation of the genome of *Autographa californica* nuclear polyhedrosis virus: a proposal. Journal of Virology 41. 1118-1121.

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

Volkman L.E. 1997. Nucleopolyhedrovirus interaction with their insect hosts. Advances in Virus research. 48. 313-348.

Volkman, L.E. 1995. Bacolovirus Bounty. Science 29. 1834.

Volkman, L.E., Blissard, G.W., Friesen, P., Keddie, B.A., Possee, R. and D.A. Theilmann. 1995. Family Baculoviridae. *In*: Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses. F.A. Murphy, C.M. Faquet, D.H.L. Bishop, S.A. Ghabrial, A.W. Jarvis, G.P Martelli, M.A. Mayo and M.D. Summers (Eds.). Vienna and New York: Springer-Verlag.

Volkmann, L.E., Summers, M.D. and H. Ching-Hsuiu. 1976. Occluded and non-occluded nuclear polyhedrosis virus grown in *Trichoplusia ni*: comparative neutralization, comparative infectivity, and *in vitro* growth studies. Journal of Virology 19. 820-832.

Washburn, J.O., Lyons, E.H., Haas-Stapleton, E.J. and L.E. Volkman. 1999. Multiple nucleocapsid packaging of *Autographa californica* nucleopolyhedrovirus accelerates the onset of systemic infection in *Trichoplusia ni*. Journal of Virology 73: (1). 411-6.

Wei, Y.J., Long, Q.X., Chen, S.W., Wang, Y.X., Zheng, J. and Wang, X.Z., unpublished.

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

Whitlock, V.H. 1974. An evaluation of the insecticidal properties of two viruses naturally associated with the bollworm, *Heliothis armigera* (Hübn.) (Lepidoptera: Noctuidae) in South Africa. Stellenbosch, University of Stellenbosch.

Wilson, A.W., J.E. Hill, J. Kuzio, and P. Faulkner. 1995. Characterisation of the baculovirus *Choristoneura fumiferana* multicapsid nuclear polyhedrosis virus p10 gene indicates that the polypeptide contains a coiled-coil domain. Journal of General Virology 76. 2923-2932.

Yang, C.L., Stetler, D.A. and R.F. Weaver. 1991. Structural comparison of the *Autographa californica* nuclear polyhedrosis virus-induced RNA-polymerase and the three nuclear RNA polymerases from the host, *Spodoptera frugiperda*. Virus Research 20. 251-264.

Zanatto, P.M., De, A., Kessing, B.D., and J.E. Maruniak. 1993. Phylogenetic Interrelationships among Baculoviruses: Evolutionary Rates and Host Associations. Journal of Invertebrate. Pathology 62. 147-164.

Zanotto, P.M., Sampaio, M.J., Johnson, D.W., Rocha, T.L. and J.E Maruniak. 1992. The *Anticarsia gemmatalis* nuclear polyhedrosis virus polyhedrin gene region: sequence analysis, gene product and structural comparisons. Journal of General Virology 73: (5). 1049-1056.

Zhang, C., Wu, X. and C. Hu. Nucleotide sequence of the polyhedrin gene of *Ectropis oblique* single nucleocapsid nuclear polyhedrosis virus. Accession number U95014.

Zuidema, D., van Oers, M.M., van Strien, E.A., Caballero, P.C., Klok, E.J., Goldbach, R.W. and J.M. Vlak. 1993. Nucleotide sequence and transcriptional analysis of the p10 gene of *Spodoptera exigua* nuclear polyhedrosis virus. Journal of General Virology 74: (6). 1017-1024.