MOLECULAR, FUNCTIONAL AND BIOLOGICAL CHARACTERIZATION OF A SINGLE ENVELOPED NUCLEOPOLYHEDROVIRUS INFECTING THE CROP PEST *Helicoverpa armigera*

by

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A thesis submitted in fulfillment of the requirements for the degree of Doctor Philosophiae in the Faculty of Sciences, University of the Western Cape.

June 2002

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KEYWORDS

Baculovirus

Helicoverpa armigera

Single-capsid nucleopolyhedrovirus

In vitro

Baculovirus repeat open reading frames (*bro*) genes

Ecdysteroid UDP-glucosyltranferase (*egt*) gene

Helicase gene

Chitinase gene

Late expression factor (*lef*) 8 gene

Phylogeny.

ABSTRACT

Molecular, functional and biological characterization of a single enveloped nucleopolyhedrovirus infecting the crop pest *Helicoverpa armigera*

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My study involves a detailed molecular and cellular characterisation of a South African isolate of a *nucleopolyhedrovirus* (NPV) isolated from the Lepidopteran insect *Helicoverpa armigera* (HaSNPV-SA). Chapter two entails the *in vitro* infection of *Heliothis zea* cell lines with HaSNPV-SA. Within this chapter I optimise the propagation of HaSNPV-SA within the cell-line HzAMI, I obtain a pure genotype (HaSNPV-Pl3) and compare the cell-lines HzAMI and Hz2E5 to determine which allows for faster propagation. Within chapter three, I discuss the identification of the ecdysteroid UDP-glucosyltranferase (*egt*) gene of HaSNPV-SA using a Hz-SNPV gene-specific probe. Studies have shown that the enzyme disrupts the ecdysteroid balance of the host larva, causing a delay in the onset of molting. That a secreted and active EGT is encoded by HaSNPV-SA was confirmed by assay of infected cell culture medium. Chapter four discusses the diversity of baculovirus repeated open reading frames (*bro*) in three nucleopolyhedroviruses of *Helicoverpa spp.* BROs of some NPV's have been shown to be involved in nucleosome organization that could block cellular replication and/or transcription and switch host machinery to viral DNA or RNA synthesis. I show that the three *bro*-genes present in four *Helicoverpa* isolates are not conserved. Furthermore, Northern analyses and/or RT-PCR indicated that all Ha-G4 3 *bro*-genes were transcribed at either 4h to 24h post infection (p.i). The final chapter, chapter five, identifies three essential baculovirus genes [*helicase*, *chitinase* and late expression (*lef*) 8] of HaSNPV-SA and uses them to confirm the placement of HaSNPV-SA in baculovirus phylogeny. All three trees confirm the placement of HaSNPV's as a group II type NPV. Based on sequence similarities, gene placement and conservation and phylogeny it is tempting to say the HaSNPV-SA isolate is a variant of HzSNPV-ELCAR.

June 2002

DECLARATION

I, declare that the work contained in "Molecular, functional and biological characterization of a single enveloped nucleopolyhedrovirus infecting the crop pest *Helicoverpa armigera*", is my own work and that I has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name ..... **Date** .....

Signed .....

This thesis is dedicated to my Mother, Father and in memory of Amma.

BIOGRAPHICAL SKETCH

Sehaam Khan was born in Cape Town, South Africa, on the 18 February 1975. She attended Accordian Primary School and matriculated at Excelsior Secondary School in 1992. Sehaam enrolled at the University of the Western Cape in 1993 and obtained a B.Sc. degree in Biochemistry and Microbiology in 1995. In 1996 she completed a B.Sc. Hons. degree in Microbiology at the same university. In 1999 she obtained her MSc. Degree in Microbiology at UWC with distinction. She is presently a full-time employer of Cape Technikon in the capacity of lecturer.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

PROF. S. DAVISON, Department of Microbiology, University of the Western Cape, who acted as supervisor, for accepting me as a student, for his enthusiasm, devotion and guidance throughout this project;

DR. BURTRAM. FIELDING AND MR. CLIFFORD. JACOBS, for invaluable discussions, technical advice and contributions to this project but, especially to Bertie for being a FRIEND;

PROF JUST VLAK, Laboratory of Virology, Wageningen University, who acted as co-supervisor for the *bro* chapter, for providing me with the opportunity of enriching my professional and personal life by allowing me to work in his laboratory in Wageningen and for his invaluable scientific input.

STAFF AND STUDENTS, Department of Biotechnology at the University of the Western Cape, especially "Club Viro", for their encouragement and assistance;

THE NATIONAL RESEARCH FOUNDATION, THE MELLON SCHOLARSHIP AND THE SOLM YACH FELLOWSHIP, for financial assistance;

MONIQUE VAN OERS, XINWEN CHEN AND MARCEL WESTENBERG, for helpful discussions and invaluable guidance;

MAGDA USMANY, ELS ROODE AND THEA VAN BEMMEL, for amazing assistance and advice;

THE "MENSA" GROUP IN WAGENINGEN, how boring life would have been without our famous talks and discussions;

SIKANDER, PRIYA AND MICHAEL, truly good friends;

WESAAL for her support, encouragement, enthusiasm and assistance whenever and wherever I needed it most, but mostly just for being "Laalie";

MY MOTHER, if I was offered all the money in the world to exchange her, IT WOULD NOT BE ENOUGH;

MY FATHER, WALEED, UNCLE RONNIE AND AMMA^{+}, for their support and encouragement;

THE LORD, WOW where to begin... for the many blessings you bestowed upon me, I recognize and appreciate each one, and for being the best friend any one person could ask for.

CONTENT PAGE

This thesis is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the *Journal of Virology* to which chapter four will be submitted for publication. Chapter three is to be submitted for publication to *Virus Genes*. Chapter five is to be submitted to the *South African Journal of Science*.

CHAPTER ONE

MOLECULAR, FUNCTIONAL AND BIOLOGICAL CHARACTERIZATION OF A SINGLE ENVELOPED NUCLEOPOLYHEDROVIRUS INFECTING THE CROP PEST *HELICOVERPA ARMIGERA*

Helicoverpa armigera:

Helicoverpa (*Heliothis*) *armigera* (Lepidoptera: Noctuidae) commonly referred to as the bollworm, is a highly polyphagous agricultural pest. Host species for *H. armigera* include important agricultural crops such as cotton, maize, chickpea, sunflower, soybean, sorghum and including ornamentals such as carnations and geraniums (Fitt, 1989). The female moth only lives for approximately two weeks, but during this period she is capable of laying up to 5000 eggs (Taylor, 1982). Once the eggs are hatched, the larvae feed veraciously on the flowing and fruiting structures of their crops, and this leads to substantial economic losses.

H. armigera is present in mainland Europe, Asia, Africa and Australia. It has been found that few pests cause as much damage as the bollworm. For many years, this pest has been successfully controlled using chemical insecticides, but the indiscriminate use of insecticides during the 1980's and 1990's, has led to the resistance of this pest to most of them. Moderate to high levels of resistance to cypermethrin, monocrotophos, endosulphan, fenvalerate and quinalphos have been recorded. Since it has been shown, that this pest has a strong ability to develop resistance to chemical insecticides, integrated pest management (IPM), which involves

the use of many techniques, including biological control, to provide effective control of crop pests, was needed.

General:

Disease-causing pathogens such as fungi, bacteria, viruses and protozoa have great potential for successful use as microbial pesticides. An important advantage of successful microbial pesticides is that it is less likely that pests would develop resistance to biological control agents. Also, because biological control is a natural phenomenon, fears with regard to environmental safety and health risks to humans and animals are less likely to arise. Beneficial insects and other non-target organisms such as parasites and predators are also not affected, due to the target specificity of the microbes.

Baculoviruses:

Baculoviruses are one of the largest and most diverse groups of insect pathogenic viruses and have great potential as biological control agents for successful use in pest control programmes. Baculovirus diseases have been described in more than 800 species of insects, which include mainly the orders Lepidoptera, Hymenoptera, Diptera and Coleoptera, but also Neuroptera, Trichoptera, Thysanoptera, Siphonophtera as well as in crustaceans (Decopoda) (Adams and McClintock, 1991). Some baculoviruses as capable of infecting few related insect species, but the majority of baculoviruses are extremely host specific. They are able to cause epizootic in nature, which appear to play a role in controlling insect populations. At present, there are numerous baculoviruses used worldwide as ecologically sound biological pesticides and there

are more than 32 registered for pest control. Table 1. mentions only some of the baculoviruses registered at present (baculovirus.com).

Table 1. Some baculoviruses registered for pest control (baculovirus.com)

COMMODITY	INSECT PEST	VIRUS USED	VIRUS PRODUCT
Apple, pear, walnut and plum	Codling moth	Codling moth granulovirus	Cyd-X (Thermo Trilogy Corp)
Cabbage, tomatoes, cotton, (and see pests in next column)	Cabbage moth, American bollworm, diamondback moth, potato tuber moth, and grape berry moth	Cabbage army worm nucleopolyhedrosis virus	Mamestrin (Natural Plant Protection)
Cotton, corn, tomatoes	*Spodoptera littoralis*	*Spodoptera littoralis* nucleopolyhedrosis virus	Spodopterin (Natural Plant Protection)
Cotton and vegetables	Tobacco budworm *Helicoverpa zea*, and Cotton bollworm Heliothis virescens	*Helicoverpa zea* nucleopolyhedrosis virus	Gemstar LC, Biotrol, Elcar (Thermo Trilogy Corp)
Vegetable crops, greenhouse flowers	Beet armyworm (*Spodoptera exigua*)	*Spodoptera exigua* nucleopolyhedrosis virus	Spod-X (Thermo Trilogy Corp)
Alfalfa and other crops	Alfalfa looper (*Autographa californica*)	*Autographa californica* nucleopolyhedrosis virus	Gusano Biological Pesticide (Thermo Trilogy Corp)
Forest Habitat, Lumber	Douglas fir tussock moth *(Orgyia psuedotsugata)*	*Orgyia psuedotsugata* nucleopolyhedrosis virus	TM Biocontrol (USDA Forest Service)
Forest Habitat, Lumber	Gypsy moth (Lymantria dispar)	Lymantria dispar nucleopolyhedrosis virus	Gypchek

Taxonomy:

Baculovirus taxonomy is based on the number of virions occluded within an occlusion body (OB) (Fig. 1), and is divided into two taxonomic genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Blissard *et al.*, 2000). In the case of NPVs, each OB or polyhedra (ranging in size from 0.15-1.5 :M) can contain as many as 200 virions while GV OB's or granule's (ranging in size from 300-500 nM), in contrast, contain a single virion and at most a few. NPV's have two morphotypes, SNPV and MNPV, depending on the single or multiple packaging of the nucleocapsids in the virion. Phylogenetic analysis based on various genes, divides the GV's and NPV's into separate groups, but also divides the NPV's into two groups, group I and II NPV's (Herniou *et al.*, 2001). *H. armigera* SNPV is a member of group II NPV.

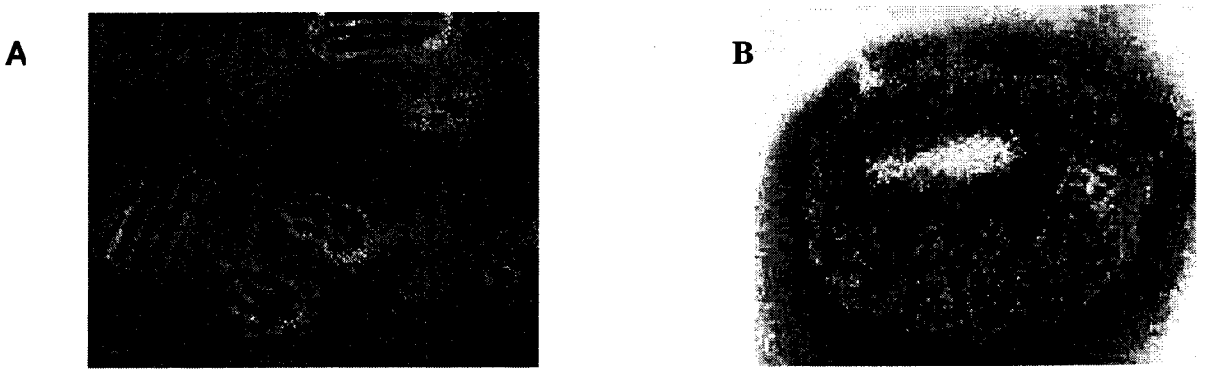

Figure 1. Electron micrographs of the two genera of baculoviruses (from www.virology.net/garryfavweb11.html and www.mioti.com/virology/bac.htm). A. *Nucleopolyhedrovirus* occlusion body (polyhedron). B. *Granulovirus* occlusion body (granule).

Two phenotypic forms of the NPV baculovirus virions exist; those virions that have been released from the OB's (polyhedra) are termed occlusion-derived virus (ODV) and the budded virion (BV), which bud from the cell and are not occluded (Fig. 2). ODV is responsible for the primary infection of the insect, whereas BV is responsible for the secondary infection within the insect. The virions are comprised of rod-shaped nucleocapsids that contain the DNA-protein

complex of the virus. The NPV nucleopcapsids are approximately 40 to 60 nm (diameter) x 250 to 300 nm (length) and comprise a 39 kDa capsid protein (Pearson *et al.*, 1988; Blissard *et al.*, 1989; Thiem and Miller, 1989 and Guarino and Smith, 1990).

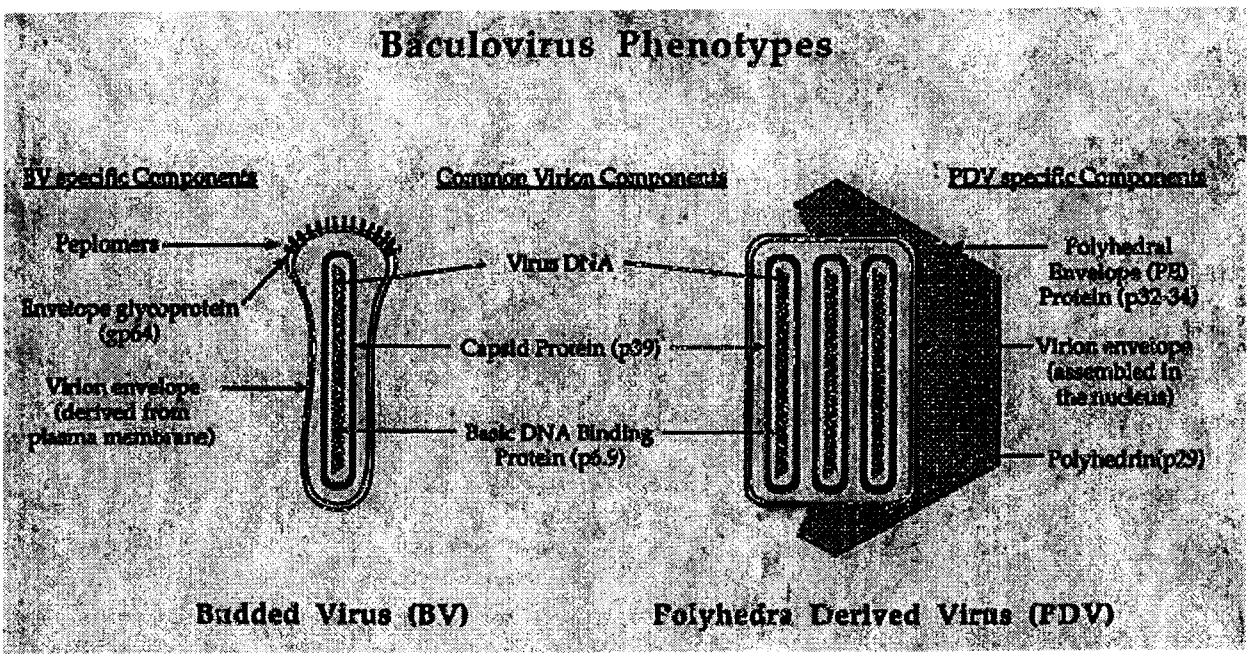

Figure 2. Schematic diagram of the two Baculovirus phenotypes, the budded virion (BV) and occluded-derived virions (ODV) (Blissard, 1996). Proteins, which are shared by both phenotypes, are indicated in the centre, and phenotype-specific components are indicated on the left for BV and on the right for ODV.

Infection cycle:

NPV's are commonly found on plant surfaces and in the soil. As is the case with the GV infection, the infection is initiated when NPV's are ingested by a susceptible during feeding of the larva. Once ingested, the occlusion body dissolves in the presence of the high alkaline pH of the insect midgut, thus releasing virions. The ODV then passes through the peritrophic membrane and fusion with the midgut epithelial cell plasma membrane commences. The pathway of NPV infection is outlined in Fig. 3. Once nucleocapsids have been synthesised within the nucleus, they pass through the plasma membrane, bud from the cell and acquire their

envelope from the plasmalemma to become budded virions. BV's are then responsible for secondary infection of surrounding cells. ODV are only formed later in the infection cycle. Studies of the *Heliothis zea* (Hz) NPV, showed virus fusion and entry into the cytoplasm, presence of a nucleocapsid in the cell cytoplasm, uncoating in the cell nucleus, BV progeny formulation and PDV progeny formation, occurred 1-4 h p.i., 2-4 h p.i., 2-4 h p.i., 8-12 h p.i., 8-48 h p.i., respectively (Granados & Williams, 1987).

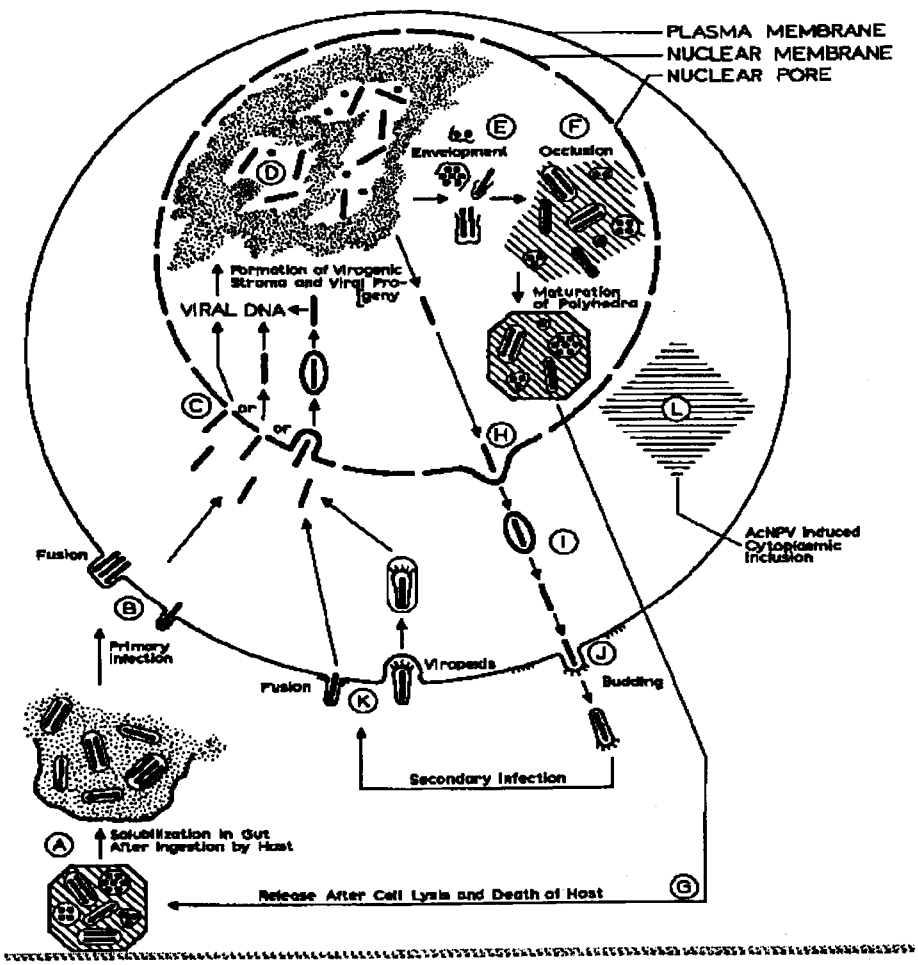

Figure 3. Schematic representation if the baculovirus infection cycle (van der Beek, 1980; van Strien, 1997). A and B. After ingestion the ODV's are dissolved within the midgut and released virions fuse with the plasma membrane. C. Virions enter the nucleus, uncoat and release viral DNA. D. Nucleocapsids are synthesised in the virogenic stroma. H, I, and J. Virions produced within the early stages of infection are released by budding and infect adjacent cells by endocytosis-K. E, F and G. During the later stages of infection, enveloped virions are occluded within the nucleus and released by lysis of the infected cell.

Symptoms:

As is the case with GV infection, typical symptoms of NPV viral infection include lethargy, loss of appetite, negative geotropism, increasing whitish appearance, and liquefaction of internal tissues. After a while complete cessation of feeding occurs, the cuticle darkens, skin becomes fragile and ruptures easily, and cadavers of infected larvae hang up side down attached by the posterior prolegs in an inverted V position known as wilting (Adams & McClintock, 1991).

Genome organisation:

As mentioned earlier, the infectious units of NPV are the nucleocapsids containing the DNA-protein complex. The supercoiled, circular, double-stranded DNA genomes are between 80 and 180 kb. Thus far 12 NPV and 3 GV genomes have been totally sequenced. These include, HaSNPV-G4 (Chen *et al.*, 2001) (NC002654); HzSNPV-ELCAR (Chen *et al.*, 2002) (NC003349); HaSNPV-N (NP 203683) (Zhang and Jin, 2000); *Orgyia pseudotsugata* NPV (OpMNPV) (NC001875) (Ahrens *et al.*, 1997); *Mamestra configurata* NPV (MacoNPV) (NC003529) (Erlandson *et al.*, 2002); *Spodoptera exigua* NPV (SeMNPV) (NC002169) (Ijkel *et al.*, 1999); *Culex nigripalpus* (CuniSNPV) (AF403738) (Afonso *et al.*, 2001); *Epiphyas postvittana* NPV (EppoNPV) (NC003083) (Hyink *et al.*, 2001); *Cydia pomonella* GV (CpGV) (NC002816) (Luque *et al.*, 2001); *Bombyx mori* NPV (BmMNPV) (NC001962) (Park, 2001); *Spodoptera litura* NPV (SlMNPV) (NC003102) (Pang *et al.*, 2001); *Plutella xylostella* GV (PxGV) (NC002593) (Hashimoto *et al.*, 2000); *Lymantria dispar* NPV (LdMNPV) (NC001973) (Kuzio *et al.*, 1999); *Autographa californica* NPV (AcMNPV) (NC001623) (Ayres *et al.*, 1994); and *Xestia c-nigrum* GV (XcGV) (NC002331) (Hayakawa *et al.*, 1999). The sizes of these

genomes range from 101 (PxGV) to 179-kb (XcGV) and contain 120 (PxGV) to 181 (XcGV) open reading frames (ORF) in the different genomes. G+C content usually ranges from 40-57%. Both strands of the genomes contain coding regions, which are functional.

Gene expression:

Baculovirus gene expression is an ordered sequence of events in which each successive phase is dependant on the previous phase (Blissard and Rohrmann, 1990) and allows for the expression of viral genes and DNA replication. The study of baculovirus replication *in vitro* has greatly simplified experiments to understand virus gene expression and replication. Studies have shown that gene expression is divided into two phases: early, late expression (Friesen & Miller, 1986). Early genes may be subdivided into immediate-early and delayed-early, whereas late genes may be distinguished as late and very late genes. Usually the expression level in each succeeding phase is higher than that of the preceding one.

Early gene transcription utilises the host RNA polymerase II complex (Friesen, 1997) and in many cases is initiated with the binding of the polymerase to a TATA box. This transcript is usually initiated within a conserved CAGT motif. Examples of the former stage include genes such as, IE-1 which is one of the first genes transcribed and enhances gene expression of other genes (Guarino & Summers, 1986), IE-N which increases IE-1 expression (Carson *et al.*, 1988), and polyhedrin envelop 38 kDa protein (PE38), a major component of the polyhedrin membrane (Krappa *et al.*, 1992), Examples of the delayed stage include a 39 kD gene which is usually detected in infected cells between 3 and 6 h p.i..