THE CHARACTERISATION OF A SOUTH AFRICAN ISOLATE OF *Cryptophlebia leucotreta* GRANULOVIRUS (CrGV)

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ABSTRACT

The false codling moth (FCM), *Cryptophlebia leucotreta*, causes widespread damage to economically important fruit crops throughout sub-Saharan Africa. Fruit are rendered unfit for consumption once they have been stung by FCM larvae. Larval infestation of fruit can lead to significant pre-harvest losses or post-harvest waste, posing a major problem to the citrus industry.

Current control of the pest includes the use of chemical pesticides. The larval form of FCM is known to be infected by a granulovirus called *Cryptophlebia leucotreta* granulovirus (ClGV). Granuloviruses are highly specific against their hosts and are harmless to vertebrates, plants and the environment. The development of ClGV into a biological control agent would offer an attractive and safer alternative for the control of this pest. A full characterisation of ClGV is required prior to the virus being disseminated into the environment. In this project, the characteristics of ClGV will be examined.

Viral DNA was extracted from infected larvae and the DNA analysed by restriction fragment length polymorphism (RFLP). Fragmentation profiles of the South African and Cape Verde (CV3) isolates of the virus were compared, revealing distinct differences between them. The size of the ClGV-SA genome was calculated to be 112 kbp, identical to the size of the CV3 isolate. Physical maps for five restriction enzymes were constructed for the ClGV-SA genome. The alignment of these maps with maps the CV3 isolate (for the same enzymes) further highlighted the differences between the isolates. The genetic engineering of granuloviruses could significantly improve the speed of kill of these viruses. Therefore essential genes like *egt* and granulin were isolated (by PCR) and their position located in the genome. Both genes were sequenced and their phylogeny with other granulin and *egt* genes investigated. Finally, the incidence of ClGV in natural populations of FCM larvae was investigated, by screening field-collected larvae for the presence of the virus. ClGV was successfully detected from dot blots of larval DNA using both radiolabelled and non-radiolabelled probes and by PCR. Trends regarding the incidence of ClGV in natural populations of larvae were also determined.

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ABBREVIATIONS

AMP- ampicillin

Bp - base pair

BSA – bovine serum albumin

BV - budded virus

CfGV - *Choristoneura fumiferana* granulovirus

ClGV - *Cryptophlebia leucotreta* granulovirus

da - daltons

DNA - deoxyribonucleic acid

dNTP - 2`-deoxynucleoside-5`-triphosphate

ds - double stranded

°C - degree Celsius

EDTA - ethylene diamine tetra-acetic acid

e.g. - example

egt - ecdysteroid-UDP-glucosyltransferase

et al - et alia (and others)

FCM - false codling moth

Fig. - figure

g - gram

GV - granulovirus

HCl- hydrochloric acid

IPTG – isopropyl thio-β-D-galactoside

kbp - kilo base pair

kda - kilodalton

L – litre

LB – Luria – Bertani medium

LoGV - *Lancanobia oleracea* granulovirus

M - molar

ml - millilitre

mM – millimolar

MNPV - multiple nuclearpolyhedrovirus

NaOH – sodium hydroxide

ng - nanogram

nm - nanometer

NOB - non-occluded baculovirus

NPV - nuclearpolyhedorvirus

OB - occlusion body

ODV - occluded virus

PCR - polymerase chain reaction

% - percent

pH - negative logarithm of hydrogen ion concentration

RE - restriction enzyme

RNA - ribonucleic acid

SDS - sodium dodecyl sulphate

SNPV - single nulearpolyhedrovirus

TAE – Tris acetate EDTA

TBE – Tris borate EDTA

TE - Tris-EDTA

Tris - tris(hydroxymethyl) amino methane

rpm - revolutions per minute

μl - micro litre

μm – micron

UV – ultraviolet

V – volts

X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER ONE: REVIEW OF LITERATURE

1.1) INTRODUCTION

A virus is defined as a set of one or more nucleic acid template molecules, normally encased in a protective coat, that is able to organize its own replication within a suitable host cell (Tanada & Kaya, 1993). The virus particle is composed of a protein shell (capsid) and contains one type of nucleic acid (DNA or RNA), which is either double or single stranded and has different modes of replication. There are more than 20 groups of viruses known to be insect pathogens. The *Baculoviridae* are a large family of occluded DNA viruses pathogenic predominantly for holometabolous insects (Blissard & Rohrmann, 1990). Baculoviruses are rod-shaped DNA viruses and are composed of two genera that are differentiated by their type of occlusion bodies. They are identified on the basis of their enveloped nucleocapsid and occlusion in a protein crystal (Maramorosch, 1977). A protein occlusion body protects their genetic material, which can be destroyed by sunlight or alkaline conditions in the insect gut. The presence of the protein occlusion body thus improves their persistence (Unknown 1, 2000). They have been identified in hundreds of insect species inhabiting forests and rivers, and in arthropods inhabiting terrestrial and marine ecosystems. As early as the 1930s baculoviruses were observed as effective biological control agents against insect pests. Baculovirology has since emerged as a dynamic and technologically important field in the last few decades (Miller *et al*, 1997). Interest in baculoviruses has centered on their natural ability to control pest populations (Blissard & Rohrmann, 1990). These viruses are the safest insect virus to use as bioinsecticides since no similar viruses are known to infect vertebrates or plants (Unknown 1, 2000). Due to their high pathogenicity and specific host range for many economically important lepidopteran insect pests, interest has been generated in the use of granuloviruses as insecticidal agents. Of specific interest is the *Cryptophlebia leucotreta* granulovirus (ClGV), which is considered to be a highly effective control agent against the false codling moth (FCM). *Cryptophlebia leucotreta* a serious pest of citrus, cotton, maize and other crops in sub-Saharan Africa (Jehle & Fritsch, 1992).

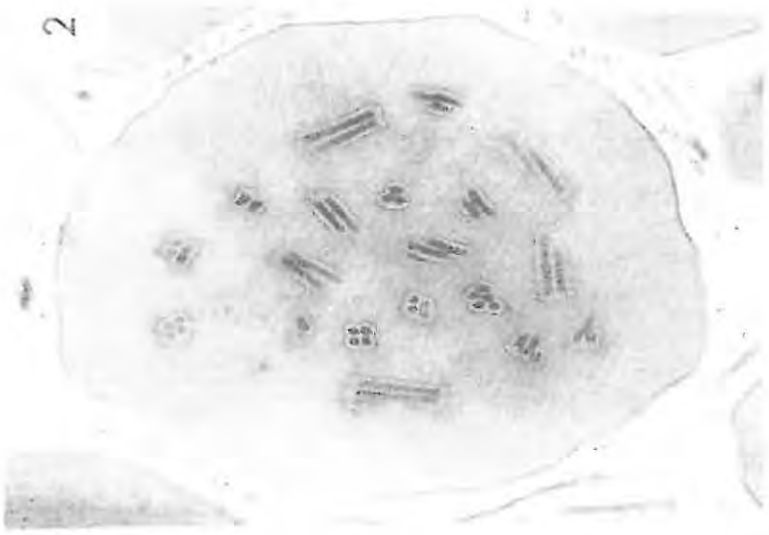
1.2) CHARACTERISTICS OF THE FAMILY *BACULOVIRIDAE*

Baculoviruses are a diverse group of large viruses with covalently closed, double stranded DNA genomes of 88-153 kbp which are pathogenic to insects (Blissard & Rohrmann, 1990). Baculoviruses, from *baculum,* refers to the shape of the virion. The family is subdivided into two genera, the genus *Granulovirus* (GV) and the genus *Nucleopolyhedrovirus* (NPV). The subdivision of the family into the two genera is based on the occlusion body morphology. NPVs (Fig. 1A) form large polyhedral occlusion bodies that contain multiple virus particles, whereas GVs (Fig. 1B) form a much smaller ovoid occlusion body that generally contains a single virion (Webster & Granoff, 1999). A feature of baculoviruses is the occlusion of the rod-shaped nucleocapsids in a crystalline protein matrix. It provides protection for the virus in the natural environment, thereby allowing certain baculoviruses to be utilized as pesticides (Maramorosch, 1977). The matrix protein (polyhedrin or granulin) is produced in large amounts late in the infectious cycle. Occlusion within the crystals stabilises the virions, enabling them to remain infectious in the environment for long periods of time. The crystals are alkali soluble, thus after their ingestion by host insect larvae, they dissolve in the alkaline environment of the insect's midgut and virions are released causing infection (Unknown 1, 2000).

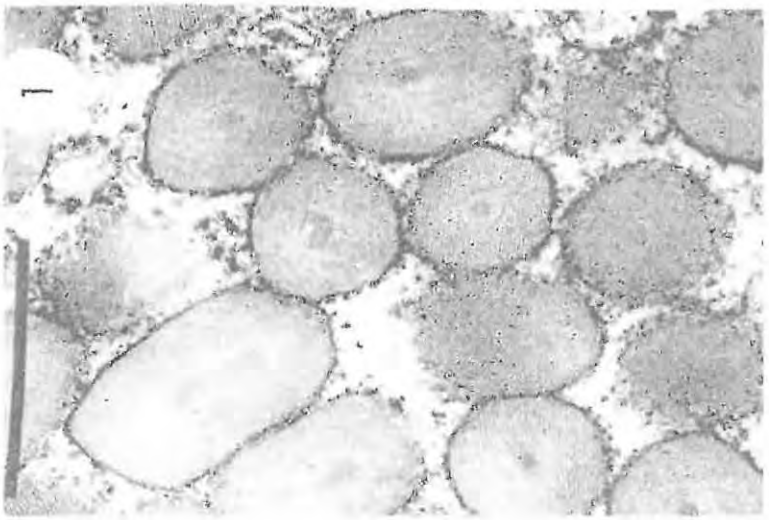
In NPVs the rod-shaped particles contain DNA and replication is confined to the cell nucleus. The occlusion bodies of the GVs are minute crystals that are found in both the nucleus and cytoplasm with only one virion per crystal (Smith, 1971). The NPVs usually exhibit a polyhedral shape and two major structural relationships – those that contain many singly enveloped virions and those, which contain bundles of nucleocapsids common to a single envelope. GVs are ovicylindrical in shape, with the inclusion body protein that surrounds the enveloped virion called granulin (Summers *et al*, 1975).

GVs are related to NPVs, but differ in the cytopathology of infected cells (Crook, 1986). The hosts ranges of baculoviruses differ since GVs tend to infect lepidopteran larvae while NPVs have a wider range of hosts in many species like the orders *Orthoptera, Trichoptera* and *Neuroptera* (Tanada & Kaya, 1993). The NPVs are highly virulent and the presence of a few particles can initiate an infection which causes the host to die within 3 to 10 days.

Baculoviruses cause host insects to die in a way to maximize the chance that other insects will come into contact with the virus and in turn, become infected, thus they have significant potential for use as control agents (Weeden, 1996).



(A)



(B)

Figure 1: The typical structures of members of the baculovirus family
(A) nucleopolyhedrovirus (MNPV) (B) granulovirus (Maramorosch, 1977)

1.2.1) Virus structure

All baculoviruses have virions of the same basic structure: an envelope, a rod-shaped nucleocapsid in which an amorphous layer exists between the nucleocapsids and envelope, a capsid, and a DNA core (Fig. 2). The evolution of the baculovirus structure appears to be in response to the unique feature of the life cycles of their invertebrate hosts. Critical to their ability to replicate and spread infection throughout the insect population, is the structure of the virion, which is present in two forms in a single infection cycle. The occluded virus (ODV) is encapsulated in a protein matrix composed of either granulin or polyhedrin while the budded virus (BV) is not occluded. The ODV is effective at infecting insects but does not spread infection in the tissues like the BV (Miller *et al*, 1997). The DNA is a single circular molecule, which is supercoiled and double-stranded. The rod-shaped baculovirus measures from 40-60 nm in diameter and 200-400 nm in length. The virions are structurally complex and contain at least 10-25 polypeptides with molecular weights ranging from 10 - 160 x 10^3 Da (Tanada & Kaya, 1993). A distinct structure on the surface of the occlusion is the capsule membrane. Another characteristic feature of the *Baculoviridae* is the intranuclear formation of virogenic stroma (an electron dense structure on the chromatin network which appears to be involved in nucleocapsid assembly) (Maramorosch, 1977).

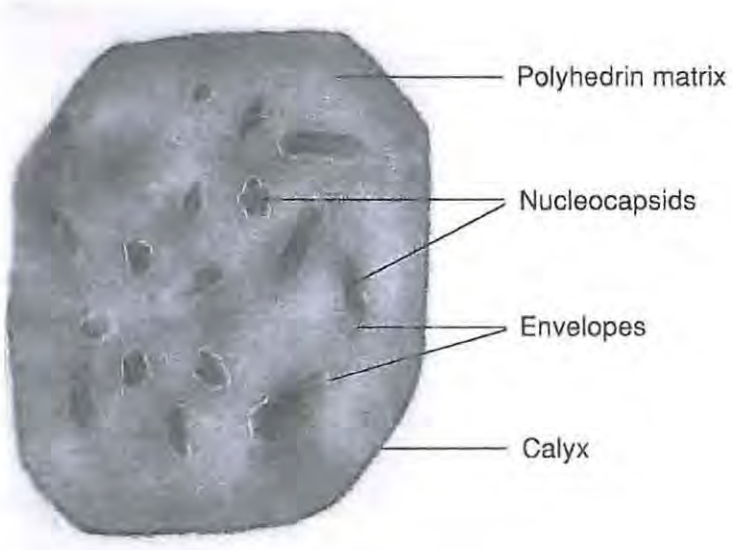


Figure 2: Baculovirus structure (O`Reilly *et al*, 1992)

1.2.2) The nucleocapsid

The nucleocapsid is similar in structure in both GVs and in NPVs. The nucleocapsid is composed of a proteinacous capsid and a cylindrical DNA-protein core. The capsid itself is made up of subunits arranged in a lattice (subunits assembled in rings stacked on top of each other). The capsid is tubular, capped at both ends and filled with DNA filaments. Microtubules are involved in the assembly of the nucleocapsids. The packaging of the DNA into the capsid is associated with the cap structure. The caps at each ends of the nucleocapsids differs morphologically. The capsid structure is composed of rings of sub-units in a stacked series. There is a polarity for orienting the nucleocapsids for viral envelopment, for attachment and penetration through nuclear pores, for emergence from the cell and for invasion through the cell membrane. The capsid has a major protein of 39 kDa (Crook, 1985). DNA associates with the highly basic protein forming a DNA-binding protein. The DNA within the capsule is in the form of a supercoil or helix. The widths and lengths of the nucleocapsids remain fairly constant for all baculoviruses (Tanada & Kaya, 1993).

1.2.3) The enveloped virion

At certain stages in virus replication, nucleocapsids are enveloped and called a mature virion. There are three types of envelopes, each of a different origin:

- An envelope produced in the nucleus (*de novo*)
- An envelope acquired from the nuclear membrane as the nucleocapsids exits the nucleus.
- An acquired envelope, as the nucleocapsids pass through the plasma membrane (cytoplasmic budding).

The envelope formed *de novo,* is involved in the occlusion of the virions in the polyhedron and capsid. The mature virion, found in the occlusion body has a true unit membrane (6-18 nm thick), consisting of a trilaminar structure with a central lipid-containing layer bounded on each side by a layer of protein. Between the nucleocapsids and the envelope, is an intermediate layer consisting of dense discs, which degenerate once the virion, is enveloped to become a mature virion (Tanada & Kaya, 1993). The envelope from cytoplasmic

budding has a distinctive cap of spikes on one end of the virion and the envelope fits loosely around the virion.

1.2.4) Infectious elements

Baculoviral infections may be initiated by:

- Isolated DNA
- Nuclear-enveloped virion
- Plasma-enveloped virion
- Occluded nuclear-enveloped virion

The four virions are phenotypes that differ in morphology, protein composition, origin of the envelopes, mode of penetration into the cell and infectivity in insect cells. The plasma-enveloped virion is the major type causing infections in the tissues of hemocoel and cultured insect cells. It enters the cell by viropexis. The occlusion bodies (OB) are the main infectious element for the horizontal transmission through the midgut of a susceptible host. The budded virus (BV), not in OBs, enter the hemocoel after cell lysis. The BVs spread from cell to cell, early in infection. The nonenveloped type is also infectious in the hemocoel (enters by endolytic pathway) (Tanada & Kaya, 1993). Fig. 3 illustrates BV and ODV structure.

1.2.5) Baculoviral proteins

The baculoviruses have large genomes, which encode over 150 proteins. It appears that no virion-associated proteins are essential for virus replication (Miller *et al,* 1997). It is likely that a variety of proteins are associated with viral envelopes or are present as nucleocapsid structural components or reside in the polyhedra. Genes encoding structural proteins appear to be located throughout the genome with no obvious pattern. Polyhedrin and granulin are proteins of about 245 amino acids and are major components of baculovirus occlusion bodies. Polyhedrin is highly conserved between NPVs (about 89 % homology has been identified between different NPVs). Lepidopteran polyhedrins show about 50 % amino acid identity with granulins. This protein is an integral part of the structure of the polyhedron envelope. p10 is another important protein, which is expressed late in the infectious cycle

but is not highly conserved (Miller *et al*, 1997). p10 is commonly found associated with polyhedrin, has a fibril structure and may function in the formation of its envelope. *p10* is a very late gene and is needed for the assembly and envelopment of the polyhedra (Miller *et al*, 1997). It encodes a polypeptide that affects nuclear disintegration in the final phases of cell death (Miller *et al*, 1997). A possible function is that p10 fibrillar bodies are involved in OB morphogenesis, specifically the formation of the calyx around the OB. VEF or viral enhancing factor is a 104 kDa protein, which facilitates GV infection by disrupting the peritrophic membrane, thus allowing the virion access to the surface of midgut cells. It has been suggested that this protein is essential in viral pathogenesis. VEF may have a dual mode of action: disruption of the occlusion bodies and increased fusion of the nucleocapsids with the midgut cells (Hashimoto *et al*, 1991). Vp39 (Fig. 3) is a protein that is a component of the capsid and is randomly distributed over the surface of the nucleocapsid (Miller *et al*, 1997). p6.9 (Fig. 3) is a late protein and the gene encodes a small, highly toxic basic DNA binding protein that is expressed at high levels very early in the late phase of the replication cycle and is found in association with the viral DNA.

egt (Ecdysteroid UDP-Glucosyltransferase) is a non-structural protein that is not essential for replication. The gene encodes a protein of 506 amino acids and the protein is secreted from infected cells. Sequence analysis shows that 18 amino acids are cleaved off the N-terminus during export. The mature protein is 60 kDa and is N-glycosylated. *egt* homologues have been identified in many baculoviruses from both sub-groups and appear to be an ancestral gene. There is also evidence to suggest that the gene may have been acquired from the insect host (O`Reilly & Miller, 1992).

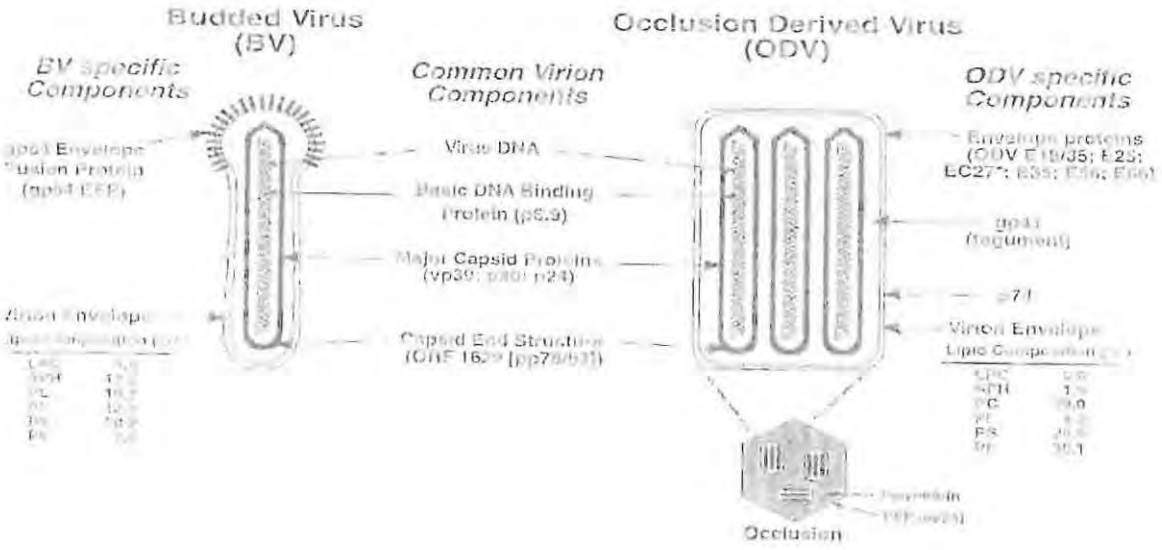


Figure 3: Budded virus and occlusion derived virus structure and location of proteins (Miller *et al*, 1997)

It belongs to a large family of UDP-glucosyltransferases, which catalyze the conjugation of ecdystreoids with UDP-glucose (O'Reilly & Miller, 1989). The *egt* product shares 21 to 22 % amino acid sequence identity with mammalian UDP-glucuronosyl transferases. In mammals the UDP-glucuronosyl transferases catalyse the transfer of glucoronic acid to a variety of exogenous and endogenous lipophilic substrates, which is essential for the elimination of carcinogens and a multitude of drugs. In insect systems the sugar conjugation reactions of this type involve glucose (O'Reilly & Miller, 1989). There is evidence that this conjugation reaction suppresses host moulting. The function of *egt* is to lengthen the time after infection that the insect feeds. Insects infected with virus with functional *egt* do not arrest feeding and thus cause lots of damage to crops. Studies have shown that insects infected with the virus lacking functional *egt* stop feeding and succumb to infection a lot sooner. They die during or soon after moulting, probably due to the stress of moulting or due to the precocious degeneration of the malphigian tubes, which accelerates moulting (Flipsen *et al*, 1995). *egt* deletions usually decrease the yield of progeny OBs while arresting host development (O'Reilley & Miller, 1989). *egt* expression

blocks the ability of the larva to pupate (enables the control of the host at an organismal level). *egt* thus impacts on the course of infection even if moulting is not affected. A possible function of *egt* is to counteract the adverse effects of ecdysteroids on viral replication at a cellular level (O'Reilly & Miller, 1989). *egt* has significant potential for use in baculovirus gene manipulation. Viruses can be genetically engineered with *egt* deletions and then used to infect insects, resulting in their cessation of feeding a lot earlier after infection. Larvae attempting to moult will have an earlier death (O'Reilly & Miller, 1992). Thus deletion of *egt* is likely to generate novel recombinant baculoviral pesticides (Miller *et al*, 1997).

Non structural genes include *p35*, an antiapoptotic gene that is expressed at high levels late in baculoviral infection. Early synthesis of *p35* is required to prevent virus-induced apoptosis. The protein encoded by the *p35* gene is 299 amino acids in length and has no recognizable sequence motifs (Miller *et al*, 1997). The *pp31* gene encodes a phosphoprotein that is produced both early and late in infection and is associated with the virogenic stroma in the nucleus (Miller *et al*, 1997).

1.2.6) Genomic organisation

A comparison of the overall gene content of divergent baculoviral genomes provides an overview of which genes are essential for virus survival. Certain genes are present in all baculoviruses while individual genes reveal how baculoviruses are changing and acquiring new distinct properties. Genome size of different members varies from 90-160 kbp, suggesting that some genomes lack genes present in other members. Nucleocapsids can expand to accommodate variations in genome content and can accept foreign genetic material. The availability of complete sequences will enable detailed comparisons of the genomes of different members. The most conserved ORFs appear to be the matrix proteins granulin and polyhedrin. Baculoviruses also carry genes that are homologous to those found in other organisms (Miller *et al*, 1997). This family of viruses appears to have acquired gp64 (Fig. 3) envelope proteins, which is related to a glycoprotein of the *Thogoto virus* belonging to the Entomopox viruses (Miller *et al*, 1997). A novel feature of baculoviruses is the presence of homologous regions (hrs). These are located throughout

the genome. They are composed of repeated sequences encompassing both direct repeats and imperfect palindromic sequences and have closely related counterparts elsewhere in the genome (Miller *et al*, 1997). These regions may play a role in DNA replication (O'Reilly & Miller, 1992), but it is also suggested that the hrs may play a function in the baculoviral lifecyle as they have been implicated as transcriptional enhancers (Miller *et al*, 1997).

Since baculoviruses have large genomes, they also encode a second class of genes. These are referred to as auxiliary genes. These genes may function at a cellular level or may facilitate replication. They also enable survival under specific conditions such as an alternate host species or cell type (O'Reilly *et al*, 1992). Examples of such genes include *polH*, which is specifically involved in OB formation. p74 associates with the envelope of the occluded virions and plays an essential role in infectivity of the midgut, the primary site of virus infection. Other genes associated with replication include *pcns* and *dnapol* (O'Reilly *et al*, 1992).

1.2.7) DNA replication

Baculovirus replication initiates a cascade of gene expression that ultimately results in the production of progeny virus. This cascade is regulated at different points during replication. Early gene expression is regulated by the interaction of cis-acting elements, viral trans acting elements and host factors. Late and very late gene expression is regulated by a combination of viral DNA replication, cis-acting elements and late viral factors. Gene products are needed for replication of DNA, e.g. a novel DNA polymerase activity is present (Mikhailov *et al*, 1986). Baculoviruses encode their own replicative proteins. Cis-acting elements are involved in the initiation of DNA synthesis. Hr elements play an important role in virus replication, as possibly the origins of replication. The presence of hrs in multiples has evolved to provide redundancy in the initiation of viral DNA replication to ensure that it occurs efficiently if one hr region is deleted (Majima *et al*, 1993). Non-hr origins of replication are necessary for maximal replication efficiency (Miller *et al*, 1997).

1.2.8) Viral membranes and envelopes

The rod-shaped particles of the granulo and nucleopolyhedroviruses are enclosed in two membranes – the inner and the outer membranes. Envelopes differ in their composition because of the different functions required of them. The baculoviral envelope is adapted for movement and infection of tissues within the insect. Baculoviruses obtain their envelopes from the host cell plasma membrane (Fig. 4). The envelope of the ODV is adapted for interacting with polyhedron structures in the occlusion process and for facilitating infection in the midgut epithelium (Miller *et al*, 1997). The ODV envelope contributes to virion stability prior to entry and infection of midgut cells. The ODV obtains its envelope within the nucleoplasm. A number of virus-encoded proteins play a role in the assembly of the envelope or act as direct precursors. This envelope consists of a lipid bilayer and it becomes tightly associated with the nucleocapsid upon occlusion. Other components of the envelope are phospholipids and a variety of proteins, which are tightly bound to the surface of the envelope. Nucleocapsids destined to be budded virus are transported from the nucleus to the cytoplasm and acquire an envelope when they bud through the membrane. They become enveloped and eventually occluded in later stages of infection. The ODVs are released after the occlusion process, upon disintegration of the insect. They then contaminate the foliage, which is subsequently ingested by other susceptible insects, thus continuing the infection process (Miller *et al*, 1997). There exists a difference in protein and lipid composition between the baculoviral and ODV envelopes. This is due to the difference in function of both envelopes the baculoviral membrane has gp 64 (which ODV lacks), that enables virus entry into cells via adsorptive endocytosis during secondary infection (Smith, 1967). Baculoviral envelopes are adapted for movement and infection of cells and the ODV envelope has proteins that assist in attaching the envelope to the microvillar membranes of the host's gut, which is the primary site of infection (Miller *et al*, 1997).

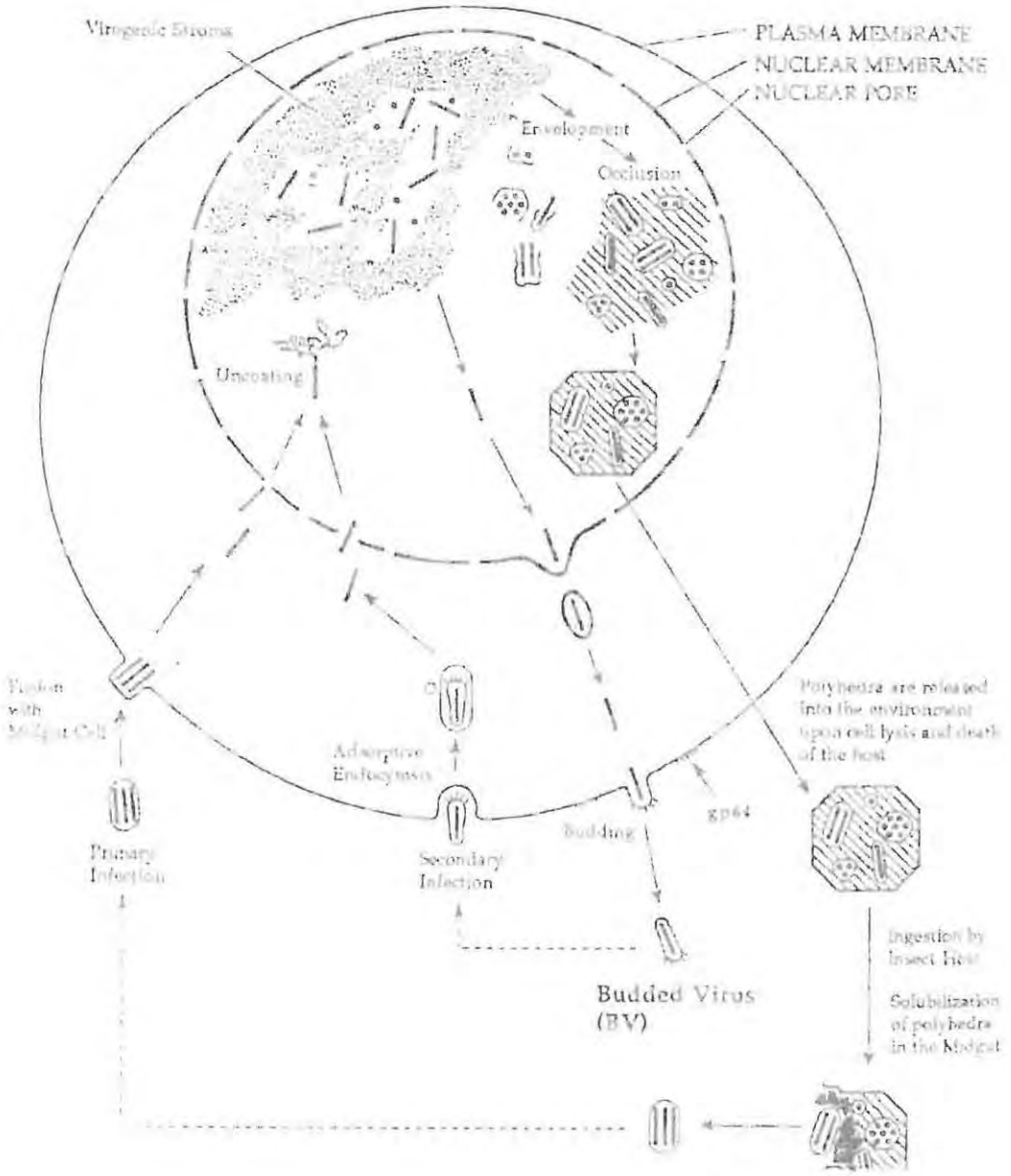


Figure 4: Replication and infection of nucleopolyhedrovirus (Rohrmann, Webster & Granoff, 1999)

1.3) BACULOVIRUS GROUPINGS

1.3.1) Nucleopolyhedroviruses

NPVs are the most common and widely distributed baculoviruses phylogentically, infecting over 400 insect species across seven insect orders (Volkman *et al*, 1995). These viruses are easily recognized because of the presence of unique polyhedral bodies in the nuclei of infected cells (Tanada & Kaya, 1993). They have hexagonal polyhedra and are about 0,5 - 1,5 microns (um) in size. Infection in hosts occurs in the adipose tissue of the hypodermis, trachea and the middle intestine (Unknown 1, 2000).

1.3.1.1) Characteristics

The two morphotypes SNPV and MNPV differ in the number of nucleocapsids per envelopes. The MNPVs are many times more pathogenic than SNPVs because of the number of enveloped nucleocapsids (Tanada & Kaya, 1993). Most of the enveloped nucleocapsids in the nucleus are occluded in the polyhedra. Polyhedra contain RNA and cellular particles. Polyhedrin (matrix protein) constitutes 95 % and virions 5 % of the polyhedra (Crook, 1986). Shapes of the polyhedra range from cuboidal to tetrahedral to irregular depending on the specific virus. The production of polyhedra is not constant but often-infected nuclei are completely filled with them (Tanada & Kaya, 1993). Polyhedra have an external surface coat, which is rich in carbohydrates and thiol and is linked to the polyhedrin matrix by proteins. This arrangement is thought to increase the stability of the occlusion bodies (Miller *et al*, 1997).

1.3.1.2) Genome

The genome is organized in a collinear manner and contains open reading frames that have amino acid sequence identities varying from 25-90 % between different baculoviruses (Crook, 1985). Baculoviruses have large genomes that must be highly condensed to be packaged within the nucleocapsids. Histones do not seem to be associated with DNA packaging. A small gene has been identified that is thought to be a DNA-binding protein. In addition, a protein of 39 kDa has been identified that appears to be a major component of the nucleocapsids of NPVs.

Transcription begins immediately after the release of viral DNA into cell nucleoplasm and these very early transcripts are grouped into a class of genes called immediate early genes (IE). Gene expression phases are (IE), structural or late, temporally delayed and hyper expressed late genes. Delayed early genes are transcribed in the presence of a number of IE gene products and they contribute to the early phase of infection until the start of viral DNA replication (Blissard & Rohrmann, 1990). Structural genes are functional during post-infection (Tjia *et al*, 1979). The temporally delayed genes are distinct in that transcription of this class is maximum by 24 hours post infection and continues until cell death. The predominant hyper expressed late genes are for the occlusion matrix protein polyhedrin (Rotel & Faulkner, 1984).

In NPVs virus replication is restricted to the midgut epithelium. The virus invades and produces occlusion bodies in the midgut epithelium nuclei. Other tissues are then invaded (Miller *et al*, 1997). Evidence suggests that the hr-genome sequence may function as the origin of DNA replication. Three open reading frames with sequence similarity to genes encoding proteins involved in DNA replication in other organisms have been identified in NPVs. These include genes with homology similar to DNA polymerase, helicase and proliferating cell nuclear antigen. Transposon elements are present in the genome. These include a retro transposon and many nonautonomous elements. Two types of transposons appear to have originated from multicopy DNA in host genome showing that an exchange of genetic information between host and viral genome occurs (Crook, 1985).

1.3.1.3) Replication

Inclusion bodies are ingested and dissolve in the gut alkali within 3 minutes to liberate the enveloped virion. High pH and proteases aid the dissolution of the OBs in the midgut (Blissard & Rohrmann, 1990). Liberated virions (ODV) pass through the peritrophic membrane, using enzymes that disrupt the structural integrity of the membrane, creating lesions that facilitate their passage and they invade columnar epithelial cells. An enhancing factor from the polyhedra also disrupts the peritrophic membrane to allow the virus through the membrane (Blissard & Rohrmann, 1990). Invasion involves the attachment and fusion of viral envelope to the membrane of columnar epithelial microvillus in the midgut. From

the site of fusion, nucleocapsids enter the microvillus. All occurs within 4 hours of ingestion. Invasion of the nucleus occurs when the nucleocapsids attach to the nucleopores and uncoating occurs thus releasing the viral DNA for viral replication. Introduction of nucleic acid into nuclearplasm initiates the eclipse period of replication. The process is terminated by the appearance of a ring zone of virogenic stroma. In the stroma, viral DNA synthesis occurs leading to viral assembly. Virus is assembled in successive stages. The viral development cycle is completed within 24 hours. Host DNA remains in its conventional chromatin form. Viral envelopes are formed when nucleocapsids associate with fibrous filaments. Envelopes are globular and abundant. They eventually form enveloped virions. Most nucleocapsids exit the nucleus via the nucleopores or bud through the nucleo-envelope where they acquire membranes. The virus is now in its budded form (BV) and they emerge from the cell, acquiring an envelope that has virus-encoded proteins, e.g. gp64 envelope fusion protein, which enables the infection of other tissues. 24-48 hours are required for the formation of polyhedra (Blissard & Rohrmann, 1990).

Virions liberated from ingested occlusion bodies infect susceptible insect tissues. Midgut epithelial cells are infected followed by midgut connective tissue and lastly the hemocoel. The enveloped virion invades by means of viropexis (Fig. 4). Once in a secondary site, two different cycles of virion production occurs. The first corresponds to the infection cycle in the midgut and leads to BV production that migrates and infects other cells. In individual cells, baculoviral production peaks at 12-16 hours. Then viral replication shifts to the occlusion phase - where occluded virions and polyhedra are produced (Granados & Williams, 1986). The baculoviruses then initiate the second phase of infection (OBs are produced). The enveloped virion invades by means of viropexis. Refer to Fig. 4 for the process.

Viral replication stages:

Latent period (0-12 hours)

Exponential period (12-72 hours)

Stationary period (48 – 72 hours)

The formation of hundreds of polyhedra filling each nucleus causes hypertrophy of infected nuclei (they become 10 times the diameter of nuclei in healthy cells) (Miller *et al*, 1997). Nuclear hypertrophy leads to the hypertrophy of infected cells and tissues and causes swollenness of the larvae. The fat body, tracheal matrix and epidermis produce the most number of polyhedra. As virus replication proceeds, physical distension and weakening of the plasmalemma caused by OB formation and production of viral protease, results in lysis of the nuclei and cells. Millions of polyhedra are thus accumulate in tissues. Polyhedra are released into the haemolymph when the basal lamina ruptures. Larvae eventually die at this point. During the occlusion phase, viral encoded chitinase is produced which enables disruption of the chitin-rich cuticle, to release polyhedra into the environment from the dead larvae (Miller *et al*, 1997).

1.3.2) Granuloviruses

The disease called granulosis was first detected by a Frenchman called Palliot in 1926, in the larvae of the white butterfly *Pieris brassicae*. This disease is found in larvae but occasionally in pupae in the order lepidoptera (Smith, 1967).

The term is applied to diseases caused by viruses, which have minute granules contained in occlusion bodies. The GVs produce virions that are biochemically and structurally similar to those of the NPVs, but the virions are occluded individually in smaller occlusion bodies called granules. GVs occlusion bodies are oval and 120-300 nm in length (Crook, 1985). Granulin is the major protein making up GVs and the gene encodes a polypeptide of 25-30 kDa. Granulin appears phosphorylated and multimers of the polypeptides form subunits that make up the crystal lattice of the occlusion bodies. The capsules are oval shaped and resistant to enzyme action during decomposition of the host larvae (Smith, 1967). They attack adipose tissue and the capsules can survive for years, out of the sunlight. Sizes and shapes of the nucleocapsids and enveloped virions of GVs are similar to NPVs. Nucleocapsids acquire envelopes by means similar to NPVs.

The two baculoviruses differ mainly in

- Number of nucleocapsids in an envelope

- Cytopathology
- Occlusion process of enveloped nucleocapsids by capsule matrix protein

Capsules are much smaller and ovoid in shape with one virion (Tanada & Kaya, 1993). Virus reproductive strategy is to produce a transient infection in the midgut epithelium. Depending on the type of GV, they then enter the fat body and invade other major tissues. Virus replicates first in the nucleus and then in the cytoplasm, after the rupturing of the nuclear envelopes (Miller *et al*, 1997). Granuloviruses are limited to the order lepidoptera with about 100 species known to be attacked by them (Volkman *et al*, 1995).

1.3.2.1) Genome

The genomes of GVs are similar to the NPVs and their sizes fall within the same range. A number of structural proteins similar to those of NPV have been isolated. GVs contain a supercoiled double stranded DNA genome. Many different genes have thus far been located on the GV genome- the granulin gene, enhancin genes, *iap* gene, DNA helicase and DNA ligase genes (Hashimoto *et al*, 2000). The *egt* gene has also been identified and sequenced (Wormleaton & Winstanley, 2001). Granulin is closely related in structure and function to polyhedrin. It has 2–3 additional amino acids and a highly conserved N-terminus. Granulin sequences are highly conserved. (Crook, 1985). The *iap* gene encodes a polypeptide of 31,3 x 10^3 Da. A region near the C-terminus of the polypeptide contains a zinc-finger like motif. The *iap* gene is similar to other genes with the ability to regulate apoptosis (Crook, 1985). Few studies on the expression of the GV genomes have been conducted (Tanada & Kaya, 1993).

1.3.3.2) Replication

The mode of infection and replication by granuloviruses, are similar to that of nucleo polyhedroviruses. Replication begins in the cell nucleus and induces the breakdown of the nuclear membranes, such that morphogenesis is completed in the cytoplasm (Crook, 1991). The highly alkaline midgut juice dissolves the ingested capsules and the liberated enveloped virions attach and fuse to the plasma membrane of the microvillus of the columnar midgut cell. Nucleocapsids enter the microvillus, migrate to the nucleus and

attach to the nucleopores within 2 – 6 hours. The difference between GVs and NPVs, is that the GV nucleocapsid injects the DNA into the nucleus at the pore. The initial phase of replication is identical to that of NPVs, with enlargement of the nuclei occurring, then the development of virogenic stroma, then hypertrophy of the nucleus and then nucleocapsid production (Miller *et al*, 1997).

Uncoating is initiated by the virion-associated kinase. Virogenesis begins in the nucleus with the production of virogenic stroma. The eclipse period is brief. Capsids appear after 6-12 hours and are incorporated with the viral nucleoprotein core. After the appearance of the nucleocapsids, the nuclear envelope breaks down and virogenesis continues in the nucleus and cytoplasm. The envelopment of nucleocapsids and their occlusion in capsules occurs in either the nucleus or the cytoplasm. The movement of virions from the midgut epithelium into the hemocoel resembles that of NPV. The infection of the fat body occurs via viropexis of nucleocapsids with peplomer envelopes. The occlusion of nuclear-enveloped nucleocapsids occurs infrequently in the midgut but occurs in other secondary sites as in NPV. As mature virions begin to accumulate, masses of granulin appear. Virions quickly accumulate and form distinct masse throughout the cell (Miller *et al*, 1997). Capsule formation occurs by granulin condensing on the viral envelope (Tanada & Kaya, 1993).

According to Crook (1985), a less common form of complete replication occurs with the formation of occluded virus entirely replicating within the cytoplasm of cells, which retain an intact and apparently normal nucleus. But it seems unlikely that GVs code for all the enzymes required for the replication of DNA (Crook, 1986). Fig. 5 illustrates viral assembly in granuloviruses.

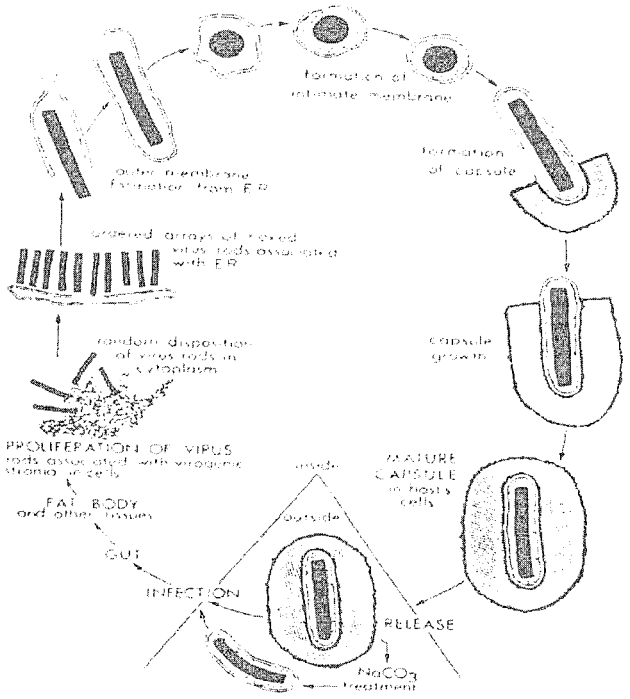


Figure 5: Viral assembly in granuloviruses (Crook, Webster & Granoff, 1999)

1.4) GENERAL EFFECTS ON THE HOST

The diverse group of baculoviruses is mainly pathogenic for invertebrates with over 600 species of insects have been reported to be infected (Blissard & Rohrmann, 1990). Primarily baculoviruses infect in the order lepidoptera and diptera. Insects killed by baculoviruses have a characteristic shiny appearance and hang limply from vegetation. They are extremely fragile to the touch and rupture easily to release fluid filled with infective virus particles. This tendency to remain attached to the foliage and then rupture is an important aspect of the virus life cycle. Infection of other insects will occur once they eat the contaminated foliage (Weeden, 1996).

1.4.1) Nucleapolyhedroviruses

NPVs infect over 400 species and susceptible hosts are found mainly in lepidoptera. (Crook, 1985). Baculoviral diseases are those of the larval stage and signs and progression of infection depend on many factors including the instar infected, infection doses, temperature and the degree of compatibility between virus and host. Infection occurs by ingestion of free and occluded virions. The period of infection from ingestion to death depends on factors like larval age, temperature, virulence and viral dosage. Death occurs in young instars within 24-72 hours after ingestion of polyhedra, with few gross signs of infection prior to death. In older instars, infection runs between 5-10 days after which death occurs, followed by the liquefaction of the larvae (Miller *et al*, 1997). The ingested virion infects columnar epithelial cells of the midgut. Most lepidoptera show no symptoms for 2-5 days. Initial symptoms are gradual changes in colour and the integument increases in opaqueness and milkiness. They become sluggish and weak. The infected larva continues feeding. The haemolymph turns milky and cloudy at advanced stages this is due to the circulation of infected haemocytes and polyhedra released into the blood as a result of lysis of cells of various tissues (Miller *et al*, 1997). They begin to swell and appear glossy (Miller *et al*, 1997). As the infection progresses, molting of the larval instars is blocked by the production of a virally encoded enzyme - UDP-glucosyltransferase. This block facilitates increasing levels of virus production. The late stages of infection are characterized by production of polyhedrin. Near the end of infection, insect stops feeding (Crook, 1985). Shortly before dying, the larva climbs to an elevated location, e.g. treetop (Fig. 6), where it dies in an inverted position. Death usually occurs in the larval stage but some survive to pupal or adult stage (Tanada & Kaya, 1993). A characteristic of baculoviral infection is the skin becoming fragile and eventually rupturing to release millions of polyhedra (Smith, 1967).

Figure 6: Characteristic appearance of larva killed by a baculovirus (Smith, 1967)

1.4.2) Effects of the granuloviruses on lepidopteron

Granuloviruses are said to infect only insects from the order lepidoptera. The symptoms of infection range from colour changes, liquefying of body contents, abnormal eating, to white liquid oozing out of the skin. The first indication of infection is in the larva, which experiences a progressive change in colour and loss of appetite. It changes from normal to a pale whitish or milky-yellow appearance especially on the ventral side. According to Tanada & Kaya (1993), the whiteness is due to the abundance of capsules in the hypertrophied fat bodies. The larvae increase in size, become opaque and mottled. At an advanced stage of infection, the larvae have a brownish discoloration and become larger, sluggish and progressively weaker.

GVs are confined to a few tissues, especially the body fat. In some granuloviruses, mitotic proliferation of uninfected cells occurs causing an enlarged bloated larva. The dead larva wilts. Fig. 7 illustrates GV infection in a larva.

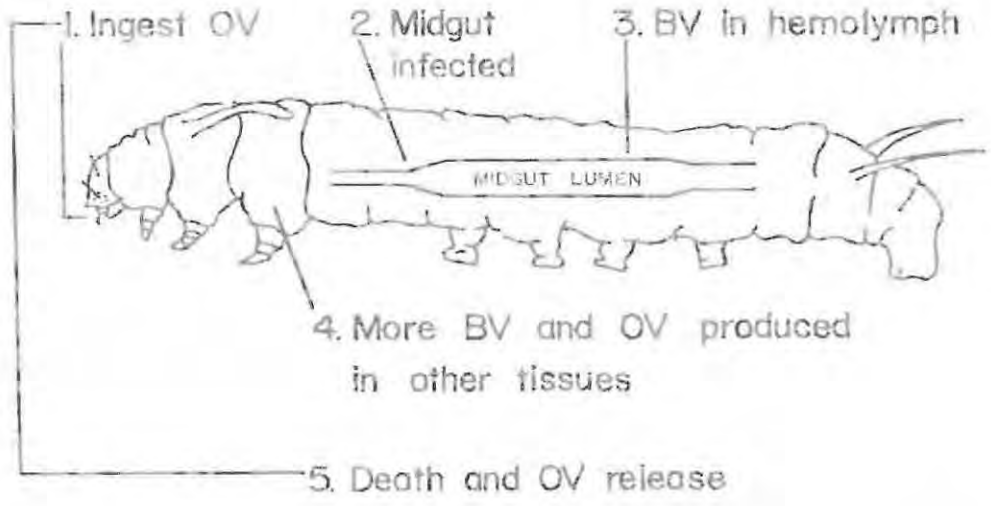


Figure 7: GV infection in a larva (O`Reilly *et al*, 1992)

The infected midgut cells are discharged into the gut lumen and virogenesis continues till cell lysis. Larvae develop diarrhoea for 2-4 days. Larval feces cause the horizontal transmission of virus (Tanada & Kaya, 1993). According to Crook (1985), in most infections, the fat body is the main site of virus replication and occlusion body production. The extent to which other tissues are infected varies, but tracheal matrix and epidermis are most commonly infected. Infection of the brain can also occur. Infection invariably leads to death of the insect after a period of a few days to several weeks.

1.5) METHODS FOR DISTINGUISHING BETWEEN DIFFERENT STRAINS OF BACULOVIRUSES

The introduction of restriction endonuclease analysis to study baculovirus genomes in the 1970s, provided a powerful means of characterizing these genomes. These analyses provide a means of estimating genome size, assessing genome heterogeneity and distinguishing between different viral strains (Miller & Dawes, 1978a). All isolates of baculoviruses can be clearly distinguished by restriction enzyme analysis (Jehle & Fritsch, 1992). Restriction endonucleases provide a means of producing discrete fragments that can be ordered to provide physical maps (Miller *et al*, 1997). Analysis of DNA restriction profiles indicate

that isolates can be placed in three subtypes. To distinguish between different strains, a physical map of the genome of the isolate must be constructed. The development of a physical map also provides an important step in elucidating gene order. In these maps, DNA fragments from RE digests are ordered on a linear or circular scale. The actual location of RE fragments or specific sequences are reported as a range within the scale of 0-100 map units. Physical maps help identify naturally occurring recombinants and illustrate genomic homology in baculoviruses (Doerfler & Bohm, 1986).

Mapping of the genome is achieved by digesting the DNA with a series of restriction enzymes, followed by Southern blotting the digest to a nitrocellulose membrane. The blot is then probed with specific fragments that are labelled radioactively or non-radioactively, to align the fragment in a specific position on the map. A southern blot shows the common DNA sequences between strains. Physical maps of genomes thus show variations and similarities between related strains of baculoviruses and this allows the subtyping of variants into groupings (Brown *et al*, 1993).

1.6) USEFULNESS IN INSECT CONTROL

"Interest in baculoviruses originally centered around their natural ability to control insect populations" (Blissard & Rohrmann, 1990). Since baculoviruses are thought to be species specific, they have been considered as candidates for biological insecticides for the control of insect pests of economic importance (Maruniak *et al*, 1984). The widespread natural occurrence of baculoviruses suggests that humans and animals have long been exposed to these viruses by contact and on their food, with no ill effects attributable to the viruses (Smith, 1967). In the 1970s, the first baculoviral product was introduced into the commercial arena. It was *Elcar Helicoverpa zea* NPV and was useful because of its high specificity and infectiousness (Tracey *et al*, 1996).

The ability of baculoviruses to protect depends on their speed of action and effective dose (that which is sufficient to initiate a productive systemic infection). Other important factors affecting their ability to protect include their stability, the amount of virus required to curb the feeding of insects and the behaviour of the insects. These factors need to be taken into

consideration, so as to control pest populations while substantially reducing crop damage (Tracey *et al*, 1996). Baculoviruses cause minimal ecological damage and are compatible with other forms of control and can be used concurrently with most chemical insecticides. The efficacy of these viruses is sometimes overwhelming, e.g. a single application of potato tuberworm has been reported to control pests equal to at least 6-10 applications of a chemical insecticide (Summers *et al*, 1975).

Crook (1985) states that in forest ecosystems, insect infestations can occur over millions of hectares and baculoviruses play an essential role in the elimination of pest populations. Field studies have shown that the efficacies of these viruses are good (Consigli *et al*, 1986). NPVs have been effective in insect control programmes. An NPV pathogenic for the velvet-bean caterpillar is used annually to treat millions of hectares of soybeans in Brazil (Summers *et al*, 1975). This virus is extremely effective since insecticides kill beneficial insects as well as pests. Granuloviruses are also useful in insect control, since they have been recorded as a natural mortality factor in many lepidopteran populations, e.g. GV diseased larvae have been reported in populations of *A. rapae* in many countries and have shown to contribute to the natural control of the pest (Crook *et al*, 1985). Transmission of virus occurs mainly by larvae feeding on contaminated remains of infected larvae. Over 80 lepidopteran species are susceptible to granuloviruses and many of these insects affect economically important crops. The use of GVs has already been successfully implemented in countries like Australia and China. The use of virus in controlling insect populations is favourable due to the rapid development of resistance by pests to insecticides. There is also concern due to the hazardous nature of insecticides on the environment. As a result, pest management using viruses as biological control agents has been encouraged (Doerfler & Bohm, 1986).

Baculoviruses play a major role in regulating the level of insect populations in the environment. To justify their usefulness in insect control, all aspects of baculoviruses and their effect on the environment and insect pests have been investigated. Epizootics can occasionally devastate some pest populations when their numbers are too high. Many insect populations are characterized by cycles of expansion and collapse. As the number of

insect's increase, insects ingest the occlusion bodies that have contaminated the soil and vegetation from previous baculovirus infections, and an infectious cycle is initiated. Transmission of virus through populations can take a few days to weeks (Miller *et al*, 1997). The surrounding vegetation is contaminated by virions upon the death of the insect. The wind, birds and other insects thus disseminate the virus. This leads to the spread of virus over large areas. The collapse of an insect population can result. Persistence, accumulation and denaturation of the virus in the environment are of critical importance. NPV and GV activity remains for long periods of time. OBs from dead larvae that adhere to host plants or contaminate the foliage and soil tend to remain there and have the ability to produce further infections (Miller *et al*, 1997).

Bioassays of soil samples in Southern Ontario, in a 2-year field study, showed active residues of *Trichopusia ni* NPV and *P.rapae* GV present in the majority of samples (Fig. 8). Soil samples from a depth of 1 cm from a field in Salisbury, USA, where no crops had been grown for 9 years, showed an average of 2,6 x 10^8 polyhedra/acre (Summers *et al*, 1975). Studies have shown that the virus may persist in soil for long periods of time (Summers *et al*, 1975).

Polyhedra and granules adhere strongly to soil particles and this natural accumulation of virus in the field has considerable value in controlling host insects. High concentrations of accumulated virus in soil have resulted in repeated epizootics. Persistence in the field is significant with regard to efficacy. Virus particles tend to persist in seasons following their application (Summers *et al*, 1975).

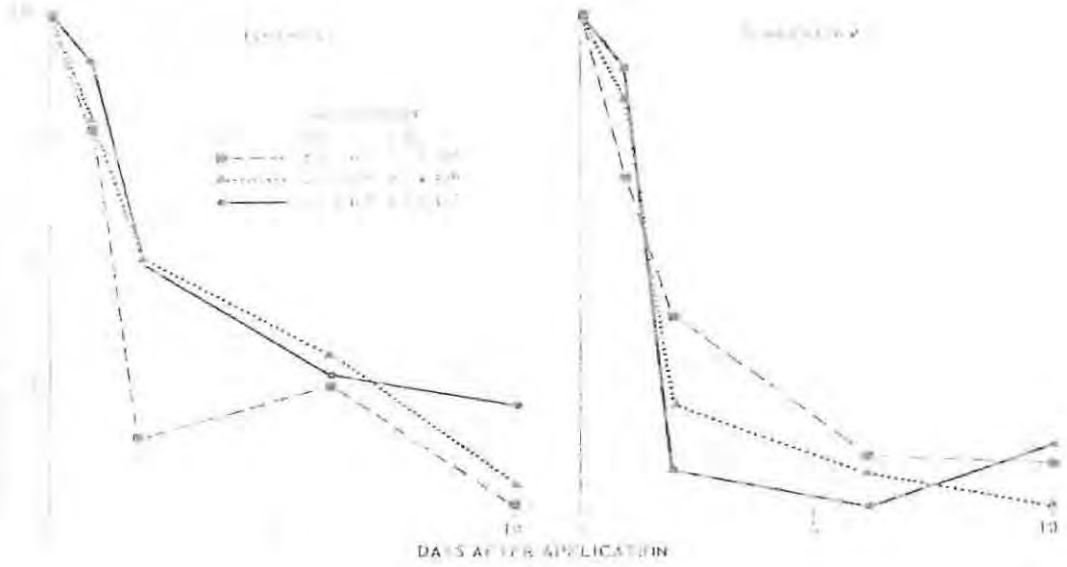


Figure 8: The activity of *Trichoplusia ni* NPV and *Pieris rapae* GV after application of virus suspensions to the leaves of cabbage plants in field plots (Summers *et al*, 1975)

The stability of the GV is dependant on the resistance of the OB protein to decomposition. Protein is broken down by weak alkalis, but withstand exposure to strong acids and other chemicals. In soil, protein should not be readily decomposed by proteolytic microorganisms (Summers *et al*, 1975). Exposure to sunlight, temperature and humidity are factors that are likely to inactivate them. But NPV and GV occlusion bodies withstand freezing and probably retain activity during prolonged exposure to normal field temperatures. Moisture on leaves does have the ability to increase the rate of inactivation of OBs by sunlight. Deposits on leaves do not wash readily from leaf surfaces. Exposure to UV light longer than 280 nm appears to be the damaging factor, since about 50 % of original activity of the virus can be lost by exposure to direct sunlight (Summers *et al*, 1975).

The safety of baculoviruses has been the focus of many studies. Certain human populations have been exposed to them for long periods of time and have shown no evidence of infection. Testing of the virus on human subjects has included inhalation, intramuscular and subcutaneous applications and injections with viral preparations. There was no observable pathogenicity or toxicity (Summers *et al*, 1975). The ability of the virus to mutate has also

been investigated. Mutations can be observed by determining the number of different hosts in which a virus can successfully replicate and then observing adaptations that the virus makes to its new host and changes in virulence. Studies in GVs have shown the specificity of the virus, since abnormal OBs (without envelopes) and abnormal functioning, have been reported when GVs infected other hosts (Summers *et al*, 1975).

These examples illustrate the usefulness of baculoviruses in controlling pest populations. Since insecticides are no longer desirable for use in crops, baculoviruses offer a safer, cheaper and more effective solution for the control of pests. Virus characterization is now required before GVs can be disseminated into the environment (Summers *et al*, 1975).

1.7) ADVANTAGES AND DISADVANTAGES OF GRANULOVIRUSES IN INSECT CONTROL

Granuloviruses, as previously stated, are increasingly used as biological agents in pest management programs. The advantages of their use have been demonstrated in field and laboratory studies. GVs are highly effective in eliminating target hosts, e.g. GV of *P.rapae* is effective against its specific hosts. In field studies, the GV of the cotton cutworm has been useful to control infestations and limit crop damage. Large-scale programs utilizing GV have already been successfully employed in countries like Australia, China and Great Britain (Summers *et al*, 1975). Unlike many synthetic chemical pesticides, baculoviruses have minimal environmental impact (Eldridge *et al*, 1992).

Baculoviruses are suitable to use in control programs because they have:

- A restricted host range and their replication limited to invertebrates
- Offer no evidence of resistance
- Kill a specific insect pest

As previously stated, the main advantage for their use is its natural control of pests. The disadvantages of GVs in insect control are that insects can sometimes survive for weeks after infection. During this time they continue feeding and still inflict damage to the crops.

If a granulovirus causes death to a pest with limited crop damage, then the virus is beneficial in the insect control process (Eldridge *et al,* 1992).

Insecticidal potential of granuloviruses is now being fully realized, but there are considerations about the overall safety with respect to their use. Considerations include production costs and effectiveness of agents in pest management programs. The disadvantages that little is known about the cellular and molecular biology of GVs. There exists very little data regarding the detrimental effects on non-target hosts. Previous studies have only investigated the effects on the whole organism. Under present safety standards, the disseminating of GV into the environment with consequent exposure to man and the environment cannot be fully investigated until the granulovirus is fully characterized and the assessment of possible interactions with non-target species both *in vitro* and *in vivo* is completed. Its characterization is also necessary to:

- Permit monitoring of insect resistance to viral strains
- Enable mutants to be detected during the production of insecticides, which may have virulence and alterations in the host range
- Allow development of assays for detecting viral residues (Doerfler & Bohm, 1986)

Baculoviruses have been reported to be safe since there have been no reported cases of infection/ disease in animals living in close proximity to local natural epidemics of virus disease of insects over historical or recent times (Summers *et al*, 1975). This has been investigated at a cellular level - deliberate attempts have been made to infect non-insect hosts and at a molecular level where animal tissues have been probed for evidence of integration of baculoviral DNA into the host's genome (Carstens, 1996). No indication of toxicity, allergic responses or pathogenicity due to baculoviruses has been reported in a study by Ignoffo & Heimpel (1965). Table 1 shows a summary of results from testing of vertebrate and invertebrate species exposed to NPVs and the comparative toxicity of an NPV with chemical pesticides. In another study men and women exposed to 6 billion polyhedra over 5 days showed no ill effects. These viruses are highly specific since organisms lacking alkaline conditions in their gut or other points of potential entry, will not

be susceptible to the virus. The occluded virus is usually applied in the field and the occlusion matrix provides an additional layer of safety in preventing virus entry into cells of non-target organisms (Miller *et al*, 1997). Baculoviruses are an excellent tool since they control specific insect pests and leave a complement of beneficial arthropod predators and parasites unharmed. They augment natural control rather than supplant it as insecticides do (Tracey *et al,* 1996).

	Response		
Material	Mammal	Bird	Fish
Heliothis NPV	Negative	Negative	Negative
Parathion	Toxic	Toxic Teratogenic Embryotoxic	Toxic
Malathion	Toxic	Toxic Teratogenic Embryotoxic	Toxic
Carbaryl	Toxic Teratogenic Embryotoxic	Toxic Teratogenic Embryotoxic	Toxic Predisposition

Table 1: Comparative toxicity of *Heliothis* NPV and three commonly used insecticides on invertebrate classes (Summers *et al*, 1975).

A disadvantage of using baculoviruses is that virulence of the virus is sometimes low and host range is restricted. The production process of the virus may be expensive and the final product may be unstable, therefore basic research is needed in gene expression, regulation, virulence and host range (Carstens, 1996). The cost would also be high, since virus is usually produced in living insects, which is a labour-intensive process.

Broad-spectrum chemicals often destroy beneficial insects and can be toxic to vertebrates; therefore granuloviruses provide an attractive, alternative means of controlling pests. A significant advantage to using them as biological control agents is the ability we have to manipulate them to improve their functioning. With the advent of genetic engineering,

insect-specific toxic genes can be inserted into the genome of a virus and this accelerates the speed that a virus kills its target insect. The speed of action of the virus may be improved by identifying and removing nonessential genes from the genome that might prolong their life in the host, e.g. deleting *egt* from the genome reduces food consumption and the larvae die faster (Tracey *et al*, 1996). Baculoviruses that carry an exogenous gene whose product is selectively toxic to insects could be safe and effective insecticides (Maeda *et al*, 1991). Baculoviruses can be used as a delivery system for foreign genes that can interfere with some critical aspect of the host's physiology or result in reduced feeding or death. Genes encoding toxins, e.g. an insect specific neurotoxin, can be introduced into the viral genome to improve the speed of kill of the virus. This has already been done where an insect toxin from a scorpion *Buthus empeus* was placed under control of AcMNPV polyhedrin gene promoter. This resulted in rapid paralysis of the larva and reduced feeding damage drastically (Stewart *et al*, 1991). An important goal of genetic engineering of these viruses would be to shorten the post infection-feeding period of the insect. Using the insect's own hormones or hormone – regulated proteins also represents a possible route for enhancing the control properties of the baculovirus (Eldridge *et al*, 1992).

Table 2 illustrates the granuloviruses that are currently in use.

Table 2: Baculoviruses that are currently in use (Weeden, 1996)

COMMODITY	INSECT PEST	VIRUS USED	VIRUS PRODUCT
Apple, pear, walnut and plum	Codling moth	Codling moth granulosis virus	**Cyd-X** (3)
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 nuclear polyhedrosis virus	**Mamestrin*** (5)
Cotton, corn, tomatoes	*Spodoptera littoralis*	*Spodoptera littoralis* nuclear polyhedrosis virus	**Spodopterin*** (5)
Cotton and vegetables	Tobacco budworm *Helicoverpa zea*, and Cotton bollworm *Heliothis virescens*	*Helicoverpa zea nuclear* polyhedrosis virus	**Gemstar LC, Biotrol, Elcar** (3)
Vegetable crops, greenhouse flowers	Beet armyworm *(Spodoptera exigua)*	*Spodoptera exigua* nuclear polyhedrosis virus	**Spod-X** (3)
Vegetables	Celery looper (Anagrapha falcifera)	Anagrapha falcifera nuclear polyhedrosis virus	**none at present**
Alfalfa and other crops	Alfalfa looper (*Autographa californica*)	*Autographa californica* nuclear polyhedrosis virus	**Gusano Biological Pesticide** (3)
Forest Habitat, Lumber	Douglas fir tussock moth *(Orgyia psuedotsugata)*	*Orgyia psuedotsugata* nuclear polyhedrosis virus	**TM Biocontrol** (2)
Forest Habitat, Lumber	Gypsy moth *(Lymantria dispar)*	*Lymantria dispar* nuclear polyhedrosis virus	**Gypchek** (1)

1.8) FALSE CODLING MOTH (FCM) - *Cryptophlebia leucotreta*

1.8.1) General description

The moth has a brown forewing (6 –9 mm) with markings and white dots (Pinhey, 1975). The hindwings are plain brown. "Leucotreta " means with white perforations. This moth is found throughout Africa, south of the Sahara and invades cultivated crops. It is severe on citrus, but also attacks many other deciduous, subtropical and tropical fruits. The navel appears to be the most heavily attacked of the sweet oranges; grapefruit and naartjies are less susceptible, while in lemons larval development is rarely ever completed, possibly due to the greater acidity and excessive juices in these fruit (Newton, 1998).

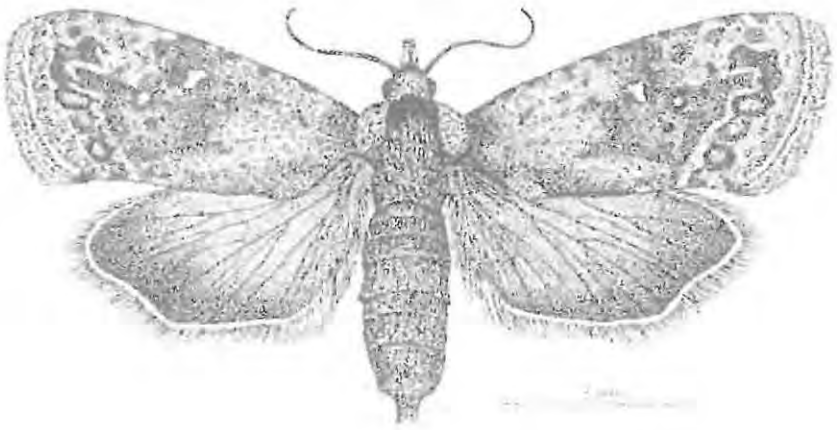


Figure 9: Description of the false codling moth (Pinhey, 1975)

1.8.2) Life cycle

The moths lay white eggs, which are oval and ridged. The eggs are about 1 mm in diameter and are frequently laid inconspicuously in depressions of the rind. As population sizes increase, more fruit are infested and more eggs are laid per fruit. The eggs are laid singly or in-groups on the surface of citrus fruits. After 1 week, the eggs hatch. The creamy larvae feed on the rest of the eggs or on other larvae. The reason for this behaviour is that rarely more than one larva completes its development in a single fruit. Thereafter, the larvae

borrow into the fruit and feeds on the inner rind and pulp but not on the juicy flesh. The larva is initially creamy white with a black head capsule, but with age takes on a characteristic pinkish-red body colour. The fruit is entered through an injury point and the last larval instar exits through a conspicous frass filled exit hole and commonly drops to the ground via a silken thread, or emerges after the fruit has fallen. This causes premature ripening of the fruit and causes it to fall. The larva then spins a cocoon on the surface of the soil. The pupal stage lasts a few days. The pupa has a transverse row of spines on each segment. The adult is a small inconspicuous moth, which is an overall dark brown. The forewings are mottled and the hindwings are paler. The male FCM is smaller and distinguished by densely packed elongated scales on the hind tibia. Females mate shortly after emergence and there is multiple mating in males and females (Newton, 1998). Adults live for 1-2 weeks and females lay up to 300 eggs (Pinhey, 1975). Eggs have a high rate of fertility. The observed peak of egg laying occurs in the first night of the oviposiotion period, which is normally 2 days after emergence. Egg laying continues for about 10-25 days and can fluctuate (Newton, 1998).

1.8.3) Effects on fruit

Under South African conditions, the moth preferentially confines itself to ripening citrus fruits during late summer. The vast majority of eggs are laid directly on the fruit surface. The emerging larvae penetrate the fruit and in citrus this leads to fruit decay, premature ripening and abscission. The small lesions caused by exploring larvae leads to fruit loss even when larval mortality is high. Infested fruit are shed in early November. Emerging larvae bore into the albedo and usually feed just below the surface. Sometimes they may tunnel through the pith to the core of the fruit. When fully grown, the larvae bore their way out of the fruit to seek a pupation site. The rind takes on a yellowish-brown colour around the point of infestation as the tissue decays and collapses. On citrus, the FCM takes 23-26 days from egg to adult. The insect will breed with a continuous supply of fruit. The first eggs will be laid in October/November; populations rise towards late summer and gradually decline with the onset of winter temperatures. There is evidence to suggest that moths originating in indigenous hosts give rise to infestations at the start of the season in some areas (Newton, 1998).

(A)

(B)

Figure 10: (A) FCM (B) a larva burrowing into a citrus fruit (Moore, 2000)

1.8.4) Control methods for *Cryptophlebia leucotreta*

False codling moth is an extremely difficult pest to control since eggs are laid continually during the fruiting period of citrus and upon hatching; the larvae bore into fruit within a few hours.

1.8.4.1) Chemicals

Protection of crops by spraying them three times with insecticide in 2,5–6 weeks before harvesting serves as a control for *C.leucotreta.* Commercial producers employ other methods using insecticides in the peaches industry (Annecke & Moran, 1982). Chemical control is difficult because of the inaccessibility of the many life stages and expensive due

to the persistent pest pressure throughout the fruiting season. Attempts to control the pest have been made with a variety of insecticides. Sprays of DDT and parathion have reduced infestations, but presented residue problems. Chitin synthesis inhibitor insecticides have also been used, but have proved to be expensive (Newton, 1998). A total amount of 5 % of fruit stung by the false codling moth is regarded as an acceptable toll. This can be decreased by orchard sanitation: stung fruits are removed and destroyed before caterpillars in them emerge to pupate in the soil. Sanitation measures are expensive and laborious and do not always guarantee satisfactory control (Newton, 1998). There are three insecticides that are currently in use. Insect growth regulators (IGR), e.g. triflumuron, work well but coverage on trees must be thorough. Most growers are unable or unwilling to apply the IGR at the required volumes. Development of resistance to this product has also been found in some areas. Another chemical in use is microencapsulated methyl parathion. However this chemical is detrimental to natural enemies and its pre-harvest interval exceeds its period of residual efficacy. The third product is Fenpropathrin, which is a pyrethroid. It is also detrimental to natural enemies and can only be applied four weeks before harvest. The use of insecticides involves high cost and has side effects; therefore, their use is limited in citrus orchards (Moore, 2000).

1.8.4.2) Viruses

The manipulation of pathogens to reduce pest populations is called microbial control. A microbial pesticide may contain a virus and functions as a pest control agent. Recombinant baculoviruses can be used in fields as microbial agents.

Microbial agents offer good control if they:

- Are specific to the target organism
- Are harmless to vertebrates and plants
- Show little/no environmental damage
- Cause no secondary pest outbreak
- Possess mass production capability with facultative pathogens

Pathogen group	Pathogen	Target insect(s)
Viruses	Codling moth GV[a,b]	Codling moth
	Plodia GV[a]	Indianmeal moth
	Autographa NPV[a]	Many lepidopteran species
	Spodoptera NPV[a]	*Spodoptera* complex
Bacteria	*Bacillus sphaericus*	Mosquito larvae
	Serratia entomophila[a]	White grubs
Fungi	*Beauveria bassiana*[a]	Many insect species
	Metarhizium anisopliae[a]	Many insect species
	Nomuraea rileyi	Many lepidopteran species
	Paecilomyces fumosa-roseus	Many insect species
	Tolypocladium cylindrosporum	Mosquito larvae
	Verticillium lecanii[a]	Aphids and whiteflies
Protozoa	*Nosema pyrausta*	European corn borer
	Vairimorpha necatrix	Many lepidopteran species

Table 3: Insect pathogens used as microbial control agents (Tanada & Kaya, 1993)

Table 3 shows that a granulovirus has already been used as a microbial agent against the codling moth.

GVs offer potential for microbial control of codling moths. It has already been demonstrated that repeated application of GV against the codling moth, *Cydia pomenella*, in apple orchards significantly reduces damage done by the pest. The GV from the false codling moth kills its insect host rapidly, making it an attractive prospect as an effective control agent. Viruses that are highly toxic to the FCM larvae can be applied to trees using conventional spray machinery. Certain additives can be included in the spray, e.g. sugars, which would act as a feeding stimulant and milk powders that could protect against degradation by UV light. Susceptibility to UV light means that the spray will be effective for 1-2 weeks depending on the weather. To keep costs down, the viral spraying needs to be timed with peak period of larval hatching. Larvae die after ingesting virus and this reduces fruit damage by about 50 %. This is not sufficient for overall fruit protection, but sometimes they die after causing only superficial stings. The overall mortality from sprays is giving major benefits in the season after the spraying. Thus the use of baculoviruses does provide a long-term biological strategy for the control of the pest, and helps to reduce high pest populations (Unknown 1, 2000).

The formulation of the virus is important. An efficient virus production scheme cannot be successful without a formulation that protects it from environmental degradation. The goal of the formulation is to preserve activity and deliver product to the target system using conventional delivery technology. The usefulness of a baculovirus can be maximized through product formulation. Optical brighteners can be used to enhance baculovirus infectivity. The addition of other inert components can improve the activity of applied doses in the field and UV screening agents can help preserve viral activity, e.g. chemical UV adsorbents like uval. Substances that prolong activity and increase effectiveness include proteinacous materials, black dyes, charcoal or a combination of these (Summers *et al*, 1975). Viral insecticides are typically wettable powders that are compatible with spray equipment. A critical factor in the performance of the baculovirus is how well the host range of the viruses matches the spectrum of insects that it must control in a crop (Ignaoffo *et al*, 1965). The success of the virus will be determined by its efficacy coupled with field persistence. *In vivo* production is the leading technology with regard to production methods. *In vivo* production has the advantage over *in vitro* production since insect rearing; diets, viral infection and harvesting are well developed. Many insect viruses are also amenable to *in vivo* production, e.g. CpGV. However, the development of recombinant baculoviruses expressing insecticidal genes will eventually see the use of *in vitro* technology to support their production (e.g. the use of stirred tank reactors for their production) (Tracey *et al*, 1996). Field-testing of viruses is also required to determine the commercial potential of the insecticide. The extent to which baculovirus formulations can protect viral activity from UV degradation and improve persistence will greatly influence the market for viral insecticides.

Granuloviruses are potentially useful as pest control agents (Doefler & Bohm, 1986). Since the granuloviruses meet the requirements for microbial control agents, they offer us an attractive alternative to spraying crops with insecticides. *Cryptophlebia leucotreta* granulovirus (ClGV) infects the false codling moth. ClGV consists of a dsDNA genome and the virions are occluded by granulin. The virus is highly specific and effective in the control of FCM. Once ClGV is fully characterized, it may be used as a pest control agent (Jehle & Fritsch, 1992). Millions of rands worth of fruit are lost annually by the citrus

industry to due pre-harvest infestation and post-harvest damage. ClGV could be sprayed on crops and cause infection in FCM larvae prior to them burrowing into fruit and causing damage. This method is beneficial since viruses are highly specific and are less harmful to the environment as compared to pesticides. Therefore ClGV is being characterized for its possible future use as an insecticide to control false codling moth damage to citrus crops.

1.9) OBJECTIVES AND RESEARCH GOALS

The objective of this project is to fully characterize the South African isolate of *Cryptophlebia leucotreta* granulovirus (ClGV). This characterization will involve establishing the size of the ClGV-SA genome, constructing physical maps for different restriction enzymes and providing restriction enzyme profiles of the DNA. Once the restriction enzyme profiles and physical maps of the ClGV-SA isolate have been established, they will be compared to the Cape Verde (CV3) isolate of the virus, to establish similarities and differences between the isolates. A plasmid library of the genome will also be established, by cloning restriction enzyme fragments of the genome into a plasmid vector. Such a library would be useful for sequencing of the genome and for isolating various genes.

Essential genes like *egt* and granulin will be isolated. Their positions in the genome will be determined and both genes will be sequenced. A final part of the project will be to probe natural populations of false codling moth larvae for ClGV using PCR and dot blot hybridizations and to compare both methods. The incidence of ClGV in natural populations of false codling moth larvae will also be determined.

CHAPTER 2: VIRUS PURIFICATION AND DNA EXTRACTION

2.1) INTRODUCTION

Many baculoviruses have been considered for use as viral insecticides (Maeda & Majima, 1990). Compared to nucleopolyhedroviruses, less is known about the molecular biology of granuloviruses and few restriction enzyme maps have been constructed (Jehle & Fritsch, 1992). Before *Cryptophlebia leucotreta* granulovirus can be used as a biological control agent against false codling moth larvae, a full characterisation of the virus is required. Thus, the virus needs to be extracted and purified from infected samples of *C.leucotreta* larvae.

Virus purification is defined as the physical separation of a virus in a concentrated form from the host cell milieu in which it has grown (Mahy & Kungro, 1996). Ultra centrifugation is the usual technique of choice for the purification of virions from their contaminating material. Virus was purified from false codling moth larvae infected with the Western Cape strain of ClGV. The extraction of virus particles involves the use of ultracentrifugation and glycerol gradients to generate virus bands. Virions are liberated from their granules with sodium carbonate and sodium dodecyl sulphate (SDS). A key step in purifying DNA involves the removal of proteins by phenol and chloroform:isoamyl alcohol. This step takes advantage of the fact that deproteinzation is more effective with two different organic solvents instead of one. A final treatment with chloroform:isoamyl alcohol alone, removes any remaining phenol (Farrell, 1993).

2.2) MATERIALS AND METHODS

FCM larvae were kindly provided by Sean Moore (Citrus Research International) from his laboratory colony that was infected with the Western Cape strain of ClGV.

2.2.1) Virus purification

Several methods for virus purification were attempted before a suitable purification procedure was obtained from Horticulture Research International. Extraction of insect material started out with homogenisation in a buffer solution that would assure a

favourable final pH and salt concentration (Frankel Conrat *et al*, 1998). Generally 2 g equivalent to 200 larvae were homogenised in 4 ml of 0.1 % SDS solution using the Sorvall Homogeniser. The volume of the homogenate was then made up to 10 ml using 0.1 % SDS and the homogenate filtered through cheesecloth to filter out the coarse tissue debris. The homogenate was then transferred to two centrifuge tubes, which were centrifuged at 10 000 rpm for 30 minutes to pellet the viral occlusion bodies. The pellets were resuspended in 6 ml 0.1 % SDS. Two continuous 30-80 % (v/v) glycerol gradients were prepared (using the Biorad gradient maker) in 0.1 % SDS in 35 ml centrifuge tubes. Glycerol was chosen for the gradients since it has no effect on the virus particles, is readily available in a purified state and produces sharp virus bands after centrifugation. The gradients were prepared by adding the 30 % and 80 % glycerol solutions to the separate mixing chambers. The homogenate was layered onto the gradients which were then centrifuged for 15 minutes at 15 000 rpm using the swing out rotor type 28 in the Beckman Ultracentrifuge. The virus bands were visualised by illumination from below (Fig. 11) and were collected by pipetting into fresh centrifuge tubes. The width of the virus band was dependant on the amount of virus layered onto the gradient. Double distilled water was added to the centrifuge tubes to fill them. The material was then centrifuged for 14 minutes at 10 000 rpm at 4°C. Two to three washes were required to produce relatively pure occlusion bodies. Each time the supernatant was discarded and the pellets resuspended in double distilled water (to the top) and spun again at 10 000 rpm for 14 minutes. After the final spin, the pellets were resuspended in 2 ml of double distilled water and stored at 4°C. Refer to Appendix 1 for the solutions used. The numbers of occlusion bodies present were counted at this point using the spectrophotometer.

2.2.2) The determination of the occlusion body number using the spectrophotometer

Once the virus had been purified, the number of occlusion bodies present in the virus sample was determined using the spectrophotometer (Beckman DU530). A 1/100 dilution, or if necessary a 1/400 dilution was made of the virus particles. Absorbance readings were taken at 350 nm and 260 nm and the number of occlusion bodies was calculated according the following formulas:

OD_{350} / 13 x dilution factor = mg/ml

OD_{260} / 13 x dilution factor = mg/ml

If absorbance readings at the two wavelengths were 0.791 and 1.907 respectively, the number of occlusion bodies would be calculated as follows:

OD_{350}: 0.791/13 x 400 (dilution factor)

= 24.34 mg/ml

OD_{260}: 1.907/13 x 400 (dilution factor)

= 25.76 mg/ml

Average = 25.05 mg/ml

Number of occlusion bodies = average x 3.83×10^{8}

$= 25.05 \times 3.83 \times 10^{11}$ capsules/ml

$= 9.5 \times 10^{11}$ capsules/ml

The formulas used to calculate the number of occlusion bodies were supplied by Dr S Wormleaton (Horticultural Research International).

2.2.3) DNA extraction

To extract ClGV DNA from 500 µl of occlusion bodies, 25 µl of a 1 M sodium carbonate solution was added and incubated for 30 minutes at 37°C. The sodium carbonate causes the occlusion body to dissolve but does not destroy or inactivate the virus particle (Harris, 1964). It was important that the solution was clear before proceeding. 30 µl of TE buffer (10 mM, pH 8) was added in order to neutralise the solution. The pH of the solution must be 8; occasionally additional TE (Tris-EDTA) buffer was required. 25 µl of RNase A was then added and the solution incubated for one hour at 37°C. SDS, a detergent that is able to solubilise the viral coat protein, was added (60 ul of a 10 % solution) along with 50 µl of proteinase K (virus degradation is facilitated by proteases). The solution was again incubated at 37°C for one hour. The isolation of intact nucleic acid material was achieved by extraction with phenol since phenol enables the precipitation of proteins to occur (Maramorosch & Koprowski, 1967). To isolate the ClGV DNA, an equal volume of buffer saturated phenol (Tris-buffered, pH 8) was added and the eppendorf was shaken vigorously by hand as vortexing can shear the DNA. This was then centrifuged in a bench top microfuge for 5 minutes at 4500 rpm. The clear upper phase was extracted, if not clear the spin was repeated. An equal volume of phenol and chloroform:isoamyl alcohol (24:1) was added to the upper phase, which was shaken again and spun at 4500 rpm for 5 minutes. The extracted upper phase was treated with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged as before. The upper phase that

was extracted was treated in one of two ways. When DNA was being extracted from large volumes of virus particles, dialysis was the method of choice for the precipitation of the DNA. This technique involved preparing dialysis membrane that was boiled in 1 x TAE buffer for 5 minutes. The upper phase was added to the bag, which was tied at one end. The dialysis bag was then clamped onto the side of a beaker containing 1 L of 1 x TAE. The dialysis occurred overnight at 4°C, with the buffer being changed the next morning and 2 hours thereafter. Refer to Appendix 1 for the solutions used. The DNA was then removed from the dialysis bag and the concentration of the DNA determined.

For smaller volumes of DNA that required precipitation, ethanol and sodium acetate were used. Two volumes of 95 % ethanol solution and a $1/10^{th}$ volume of 3 M sodium acetate were added to the DNA. The solution was mixed and incubated at –20°C for 20 minutes to overnight. The DNA was then centrifuged in a microfuge at 13 000 g for 20 minutes. The supernatant was discarded and the pellet washed with 1 ml of 70 % ethanol. The pellet was centrifuged at 13 000 g for a further minute and then allowed to dry. Once dry the DNA pellet was resuspended in 20 μl TE buffer (10 mM, pH 8).

On occasion, the DNA appeared to be contaminated with possible proteins since restriction enzymes were unable to cut the DNA. In these instances, the DNA was further purified using Genomic Tips (Qiagen), which are a reliable method for the isolation of pure high-molecular weight genomic DNA. This simple purification procedure is based on the selectivity of the Qiagen anion-exchange resin (Qiagen Handbook, 1997). To remove any remaining contaminants 50 μl of ClGV DNA was used and the volume made up to 1 ml with solution 1. Genomic tip 20/G was used and the tip was equilibrated with buffer QBT, which is emptied by gravity flow. The DNA was vortexed for 10 seconds at 13 000 g and then applied to the tip by gravity flow. The tip was then washed three times with 1 ml of buffer QC in order to remove all contaminants. The DNA was eluted from the tip with 1 ml of buffer QF (pre-warmed to 50°C in order to increase the yield of DNA recovered). 1.4 ml of isopropanol (room temperature) was then added to precipitate the DNA. This was carefully mixed and then microfuged for 2 minutes at maximum speed. The supernatant was removed carefully. The DNA pellet was washed with 1 ml of 70 % ethanol (-20°C), vortexed

and centrifuged again for 5 minutes. Once the supernatant was removed, the pellet air-dried for 10 minutes. The DNA was then resuspended in 100 μl of 10 mM TE buffer (pH 8) (Qiagen Handbook, 1997). Refer to Appendix 1 for the solutions used.

2.2.4) DNA quantification

For large scale DNA extraction (volumes of 5 ml) dialysis was used, while for volumes of less than 1 ml DNA was precipitated with 95 % ethanol and 3 M sodium acetate. Once the DNA was resuspended in TE buffer (10 mM, pH 8), the concentration of the DNA was determined using a Genequant (Pharamicia Biotech) or Beckman DU 530 spectrophotometer.

2.2.4.1) Quantification of DNA using the genequant

A 1/100 dilution of the ClGV DNA was made using triple distilled water. The absorbance of the DNA was read at 260 nm and the concentration of the double stranded DNA was calculated by the Genequant.

A good sample of DNA generally yielded approximately 30 μg/ml of DNA.

2.2.4.2) Quantification of DNA using the spectrophotometer

The concentration of DNA can be measured spectrophotometrically at 260 nm using the formula that 1 OD_{260} unit = 50 μg/ml. A 1 in 200 dilution was made of the samples. Purity was estimated by measuring the absorbance at 260 and 280 nm, with the ratio OD_{260}/OD_{280} being 1,8 if the DNA was not contaminated by proteins (Mahy & Kungro, 1996).

2.2.5) Single insect purification

A single larva was homogenised in an Eppendorf in 0.5 ml of 0.1 % SDS and centrifuged at low speed for 10 seconds. The supernatant was transferred to a fresh tube. 0.3 ml 0.1 % SDS was added to the pellet and the pellet was resuspended by vigorous mixing. This was centrifuged as before and the supernatant from this step and the previous step were combined. 30-80 % glycerol gradients were prepared in 0.1 % SDS and the supernatant was layered onto the gradients. The gradients were then ultracentrifuged using the SW 28 swing out rotor for 20 minutes at 15 000 rpm. The capsule band was recovered to centrifuge tubes, which were then filled with water. The capsules were pelleted by centrifuging for 15 minutes at 15 000 rpm. The

supernatant was removed carefully so that the pellet was not disturbed. 1 ml of water was added to the pellet, which was resuspended and transferred to an Eppendorf tube. The purified capsules were pelleted by centrifugation for 3 minutes at maximum speed in a bench top microfuge. The supernatant was removed and the pellet resuspended in 100 μl of water and stored at –20°C. Once the occlusion bodies were purified, DNA could be extracted as in 2.2.3. The DNA was precipitated using 3 M sodium acetate and 95 % ethanol.

2.3) RESULTS

2.3.1) Virus purification

ClGV was purified using ultracentrifugation in glycerol gradients. A virus band was obtained in the centre of the gradient and could be viewed by illumination from below. The band containing ClGV occlusion bodies was recovered by pipetting. Another band was generally present below the virus band, containing unknown material. Fig. 11 illustrates the ClGV virus band obtained after ultracentrifugation on a 30-80 % glycerol gradient. A typical yield from 2 g of infected insects was 8 x 10^{10} capsules/ml as determined spectrophotometrically.

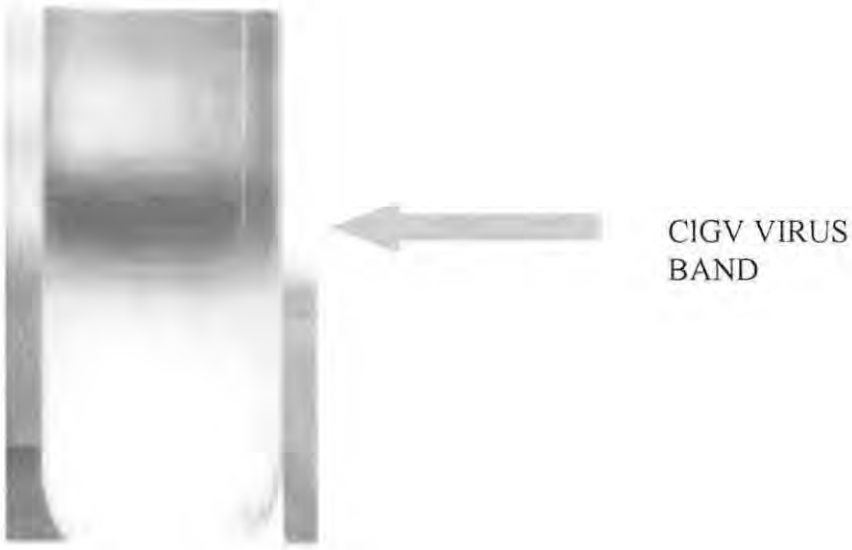


Figure 11: The ClGV virus band on a 30 80 % glycerol gradient.

2.3.2) DNA visualisation

Electrophoresis through agarose is a standard method used to separate, identify and purify DNA fragments. This simple technique allows the presence of DNA to be determined directly using staining with a fluorescent intercalating dye ethidium bromide (Sambrook *et al*, 1989). The presence of ClGV DNA was also determined by electrophoresing samples of DNA on 1 % agarose gels for 1 hour at 100 V in the presence of 0.5 :g/ml ethidium bromide. The gels were then visualised under ultraviolet light and bands on the agarose gels indicated the presence of DNA. Generally, digestion of the ClGV DNA was performed using restriction endonucleases once an adequate concentration of DNA was obtained, and these digests were then electrophoresed in agarose gels to confirm the presence of DNA. Fig. 12 shows a typical 1 % agarose gel with ClGV DNA digested with restriction enzymes thus confirming the presence of viral DNA.

1 – marker 9/*Pst*1
2 – *Eco*R1 digest
3 – *Kpn*1 digest
4 – *Xho*1 digest

Figure 12: ClGV DNA digested with restriction enzymes *Eco*R1, *Kpn*1 & *Xho*1 on a 1 % agarose gel. See Table 6, page 62 for the sizes of the molecular weight markers.

DISCUSSION

The techniques employed for virus purification and DNA extraction proved to be successful in isolating viral material from false codling moth larvae.

Virus could be adequately purified from larvae using glycerol gradients, ultra centrifugation and several washing steps. Glycerol gradients appeared to be more satisfactory for use as a density gradient support medium, since sharper virus bands were obtained than with sucrose gradients. The extraction of ClGV DNA was done successfully by both dialysis and precipitation with ethanol and sodium acetate. The key steps in the extraction process included the treatment of capsules with sodium carbonate, which dissolved away the virus protein in order to liberate the DNA (Smith, 1971). The treatment of the DNA with phenol and chloroform:isoamy alcohol was essential in eliminating contaminating proteins. Once these were added to the virus, they were mixed vigorously (not vortexed) in order to liberate the DNA. The detergent SDS and the protease Proteinase K were also essential for the breakdown of the virus particle such that its nucleic acid could be isolated. Despite the success of the techniques, contaminating material sometimes accompanied the DNA, preventing its digestion by restriction enzymes e.g. *Eco*R1. The Genomic tip (Qiagen) has proved to be useful in eliminating such contaminants.

Using these techniques adequate samples of ClGV and its DNA could be extracted for use in different molecular techniques for the mapping, profiling and overall characterisation of the virus.

CHAPTER THREE: THE ANALYSIS OF *Cryptophlebia leucotreta* granulovirus (ClGV) DNA

3.1) INTRODUCTION

Before *Cryptophlebia leucotreta* granulovirus (ClGV) can be utilised as a biological control agent, a full characterisation of the virus is required. The characterisation of the virus involves determining the size, restriction enzyme profiles and physical maps of the genome. DNA restriction enzyme profiles can be used to identify and compare baculovirus isolates (Marunaik *et al*, 1984). Electrophoresis of fragments produced by digestion of different GV DNAs with restriction enzymes show clear similarities and differences between different viruses (Crook, 1981). Similarities between profiles from different viruses can also be used to subtype variants into groupings, which may reflect their geographical origins (Smith & Crook, 1988).

The conformation and size of viral DNA has been studied by a number of techniques including sedimentation in sucrose gradients and by restriction enzyme (RE) digests. Recent studies have shown the usefulness of RE analysis of large baculoviral genomes (Crook *et al*, 1997). RE fragment patterns of DNA have been utilised to estimate genome size, to identify viral isolates and to distinguish between closely related genomic variants. Cleavage sites of a number of restriction enzymes have been localised on the genomes in order to construct physical maps of the viral DNA. For analysis of the structure and organisation of the genome, DNA has to be digested with several enzymes both alone and in combination. The analysis of RE profiles reveals a large degree of genetic diversity between virus isolates (Tweeten *et al*, 1980).

In this chapter the characteristics of the ClGV genome will be examined. These include the size of the ClGV genome, the profiles generated from single and double digests, the comparison of the South African isolate of ClGV with a Cape Verde isolate and the construction of a plasmid library of the ClGV genome.

3.2) RESTRICTION ENZYME PROFILES

Restriction endonucleases recognise specific base sequences on a DNA molecule and makes one cut in each strand, thus cleaving the molecule. Each GV DNA has unique RE patterns for each particular enzyme (Dwyer & Granados, 1987). Thus far the DNAs of granuloviruses have been analysed using a wide range of restriction enzymes (Crook, 1986). Jehle & Fritsch (1992) had constructed RE profiles for the Cape Verde isolate (ClGV-CV3) using 10 different enzymes. The same set of enzymes was selected for use in characterising South African isolate (ClGV-SA). Optimum digestion conditions, electrophoresis time and agarose gel concentrations had to be determined for each enzyme digest for optimum band separation and the resolution of small and large fragments. All isolates can be clearly distinguished by restriction enzyme analysis (Jehle & Fritsch, 1992).

3.2.1) Materials and methods

ClGV-SA was isolated from a laboratory colony of *C.leucotreta*. Larvae for virus and DNA extraction were kindly supplied by Sean Moore (Citrus Research International). ClGV-CV3 was isolated from larvae supplied by Dr S Wormleaton (Horticultural Research International).

3.2.1.1) Single enzyme digests

ClGV DNA was extracted and its concentration determined spectrophotometrically. Ideally 800 ng of DNA was required per digest. Various restriction enzymes were selected and digests were performed as follows:

800 ng	ClGV DNA
2 µl	Restriction endonuclease (20 units)
15 µl	Buffer (10 x)
x µl	distilled water
150 µl	

The digest was mixed and then incubated at 37°C for 2 hours. Fragments generated from digestion were detected by agarose gel electrophoresis. 6 x loading buffer was added after digestion to each digest ($1/6^{th}$ of the total volume). To allow for both small and large fragments of the digests to be resolved, the fragments were electrophoresed on two separate gels – a 1 % TBE agarose gel for small fragments and a 0.7 % TAE gel for the larger fragments. Refer to Appendix 2 for the solutions

used and gels made. The 1 % gel was electrophoresed at 100 V for an hour and a half in 0.5 x TBE buffer. The molecular weight marker λ*Bst*E II was used. The 0.7 % gel was electrophoresed overnight (approximately 16 hours) at 35 V in 0.5 x TAE buffer. Two molecular weight markers were run on this gel – λ*Hind* III and λ Mix 19 (used to determine very large fragment sizes). The 0.7 % gel had to be stained after electrophoresis in 200 ml of 0.5 x TAE buffer containing 20 μl ethidium bromide (10 mg/ml). The gel was stained for 30 minutes and then rinsed twice for 15 minutes in 200 ml of distilled water. The 1 % gel did not require staining since the ethidium bromide was added to the agarose. Gels were then photographed using the Kodak digital camera (DC120).

3.2.1.2) Double digests of ClGV DNA

A double digest involves digesting DNA with enzymes in pairs (Smith & Crook, 1988). Often digestion of DNA with single enzymes produced profiles where some of the DNA remained unrestricted. To further characterise ClGV and to obtain a more reliable estimate of the molecular weight of the genome, the DNA had to be digested to completion by combinations of restriction enzymes. In such digests the majority of the high molecular weight fragments observed in the single digests (whose sizes are difficult to calculate) would be cleaved into smaller products (Tweenten *et al*, 1985). Information obtained from the double digests is also essential for the construction of the physical maps for each enzyme.

Digests were performed under the same conditions as the single digests except that DNA was digested using pairs of restriction enzymes. Some examples of pairs used were *Sac*1+*Kpn*1, *Bam*H1+*Sac*1, *Sac*1+*Xho*1 and *Bam*H1+*Xho*1. Each combination of enzymes had a common restriction enzyme buffer. The digests were set up as follows:

800 ng	ClGV DNA
2 μl	Restriction enzyme 1 (20 units)
2 μl	Restriction enzyme 2 (20 units)
15 μl	Buffer (10 x)
x μl	distilled water
150 μl	

Digests were incubated for 3 hours at 37°C and then separated on agarose gels as was done for the single digests. The same molecular weight markers were used and the gel

resolving the large fragments had to be stained as was done previously. All gels were then photographed using the digital camera (Kodak DC120).

3.2.1.3) The comparison of RE profiles – ClGV-SA and ClGV-CV3

Restriction enzyme profiles from different isolates reflect if the isolates are distantly or closely related. Similarities between profiles from different isolates can also be used to subtype variants into groupings that reflect their geographical origins (Crook, 1985, 1986). A comparison between the ClGV-SA and ClGV-CV3 was made using restriction endonucleases to investigate how similar the two isolates are, as the clearest distinction between isolates is achieved by analysing their DNA with restriction enzymes (Crook, 1981). This study is necessary to assess the relatedness of the two isolates. A more precise way of relating these two isolates would also be to construct physical maps for a number of restriction enzymes (Possee & Kelly, 1988).

ClGV DNA was extracted for both isolates and digested with enzymes *Bam*H1, *Eco*R1, *Kpn*1, *Sac*1 and *Xho*1. Fragments were then electrophoresed on a 1 % TAE gel, which was then stained with ethidium bromide (10 mg/ml). The same conditions for staining were used as for the single and double digests. The restriction digests of both virus genomes were run simultaneously to highlight the variation as suggested by Possee & Kelly (1988). The digests were set up using the same protocol that was used for the single enzyme digests.

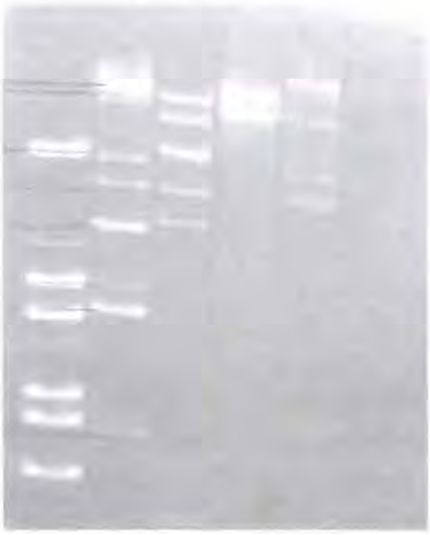
3.2.2) Results

3.2.2.1) Single enzyme digests

The restriction enzyme profiles of ClGV-SA DNA digested with enzymes are presented in Figs. 13 & 14. The resolutions of the large fragments are presented in Fig. 13 and the small fragments in Fig. 14. Results are also presented in Table 4.

1 – λ/*Hind* III marker
2 - λ Mix 19 marker
3 –*Bam*H1 digest
4 –*Eco*R1 digest
5 –*Kpn*1 digest
6 - *Xho*1 digest
7 - *Sac*1 digest

(A)



1 – λ/*Bst*E II marker
2 - λ/*Hind* III marker
3 –*Kpn*1digest
4 –*Sac*1digest
5 –*Xho*1digest

(B)

Figure 13: (A) Restriction enzyme profiles of large fragments of ClGV-SA DNA digested with *Eco*R1, *Kpn*1, *Sac*1, *Bam*H1 & *Xho*1 on a 0.7 % agarose gel. (B) Shows the improved separation of the *Sac*1 fragments. Molecular weight markers λ/*Hind* III, λ/*Bst*E II and λ Mix19 (Table 6) were included.

All the large fragments from each RE digest of ClGV DNA could be visualised. A good separation of these fragments occurred on the 0.7 % agarose gels, but digests were also run on 1 % agarose gels in order to resolve smaller fragments.

(A)

1 – λ/*Bst*E II marker
2 & 4 – *Bam*H1 digest
4 & 5 –*Eco*R1 digest

(B)

1 – λ/*Pst*1 marker
2 –*Eco*R1 digest
3 –*Kpn*1 digest
4 –*Xho*1 digest

(C)

1 – λ/*Bst*E II marker
2 –*Bam*H1 digest
3 – *Eco*R1 digest
4 –*Kpn*1 digest
5 - *Xho*1 digest

Figure 14: The small fragments of ClGV-SA DNA digested with *Eco*R1, *Kpn*1, *Bam*H1 & *Xho*1 resolved on 1 % agarose gels. Markers λ/*Pst*1 and λ/*Bst*E II are included for the calculation of molecular weights of the fragments (Table 6). Different gels are shown since optimum separation was not obtained on a single gel.

3.2.2.2) Double digests of ClGV DNA

The very large sized restriction fragments of ClGV-SA were cleaved into smaller products using double digests. From these double digests an accurate estimation of the genome size of ClGV could be made. Digests were electrophoresed on both 0.7 and 1 % agarose gels to allow the resolution of all fragments (Figs. 15 & 16). Many different combinations of enzymes were used, but for the purposes of mapping the ClGV genome B*am*H1, *Kpn*1, *Sac*1 and *Xho*1 were used in combinations. A good separation of the large fragments (Fig. 15) as well as the small fragments (Fig. 16) was obtained.

1 – λ Mix 19 marker
2 - λ/*Hind* III marker
3 –*Bam*H1/*Sac*1 digest
4 –*Bam*H1/*Xho*1 digest
5 –*Kpn*1/ *Sac*1 digest

(A)

1 – λ/*Bst*E II marker
2 - λ/*Hind* III marker
3 – *Bam*H1/*Sac*1 digest
4 – *Kpn*1 / *Sac*1 digest
5 - *Xho*1 / *Sac*1 digest
6- *Xho*1/ *Bam*H1 digest

(B)

Figure 15: Electrophoresis of large fragments on 0.7 % agarose gels after digestion of ClGV-SA DNA with pairs of restriction endonucleases. Different gels are shown in (A) and (B), as optimum separation of all fragments on a single gel was not obtained.

1 – λ/*Bst*E II marker
2– *Bam*H1/*Sac*1 digest
3 –*Kpn*1 / *Sac*1 digest
4– *Xho*1 / *Sac*1 digest
5 - *Xho*1 / *Bam*H1 digest

(A)

1 - λ/*Bst*E II marker
2 –*Bam*H1/*Sac*1 digest
3– *Bam*H1/ *Xho*1 digest
4 –*Kpn*1 / *Sac*1 digest

(B)

Figure 16: Agarose gel electrophoresis of small fragments on 1 % agarose gels after double digestion of ClGV-SA DNA. (A) & (B) are shown since optimum separation of fragments were not achieved on a single gel.

3.2.2.3) The comparison of ClGV-SA with the Cape Verde isolate of ClGV-CV3

The RE digests of both ClGV-SA and ClGV-CV3 were done using five restriction enzymes *Bam*H1, *Eco*R1, *Kpn*1, *Sac*1 and *Xho*1. The digests were run alongside one another on a 1 % agarose gel (Fig. 17). A comparative sketch was made of the RE profiles of the two isolates (Fig. 18). It can be noted that for all five enzymes there are differences between the ClGV-SA and ClGV-CV3 isolates in their RE profiles. Arrows point to fragments that are present in the SA isolate but are absent in the Cape Verde isolate (Fig. 18).

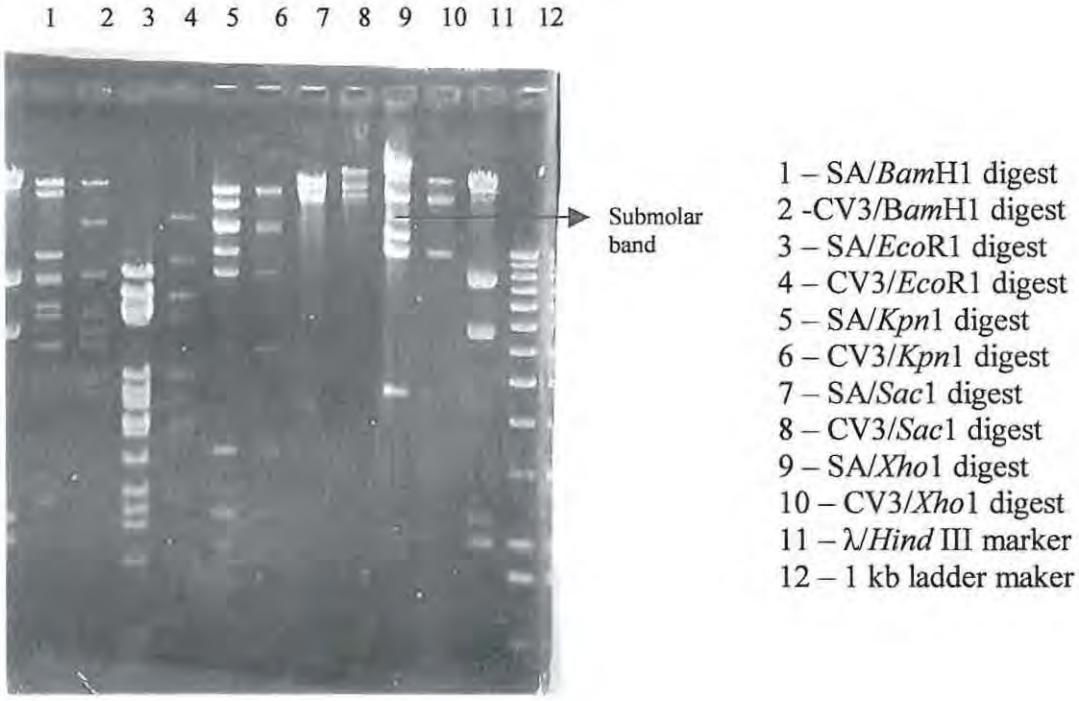
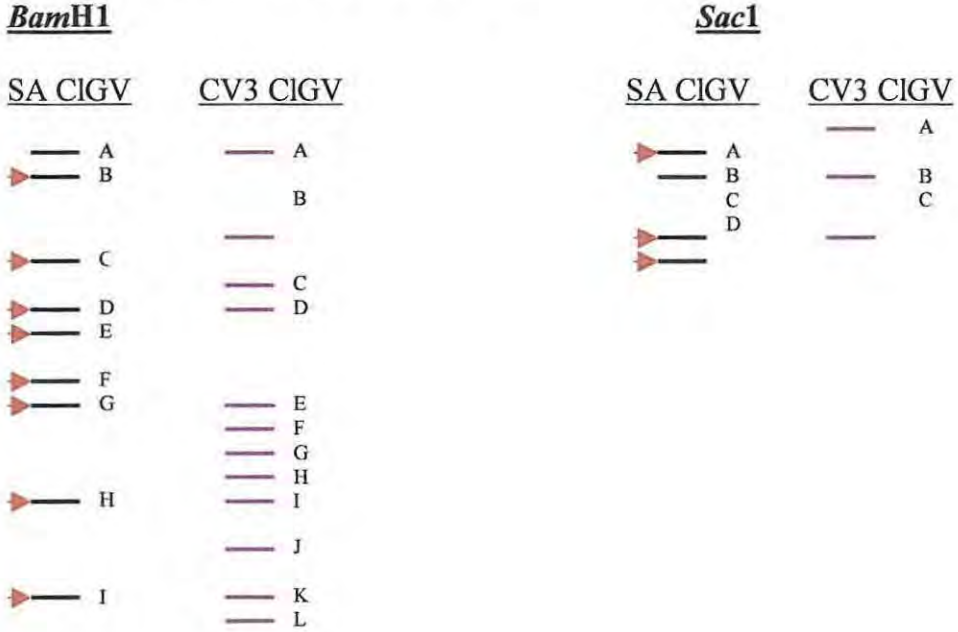
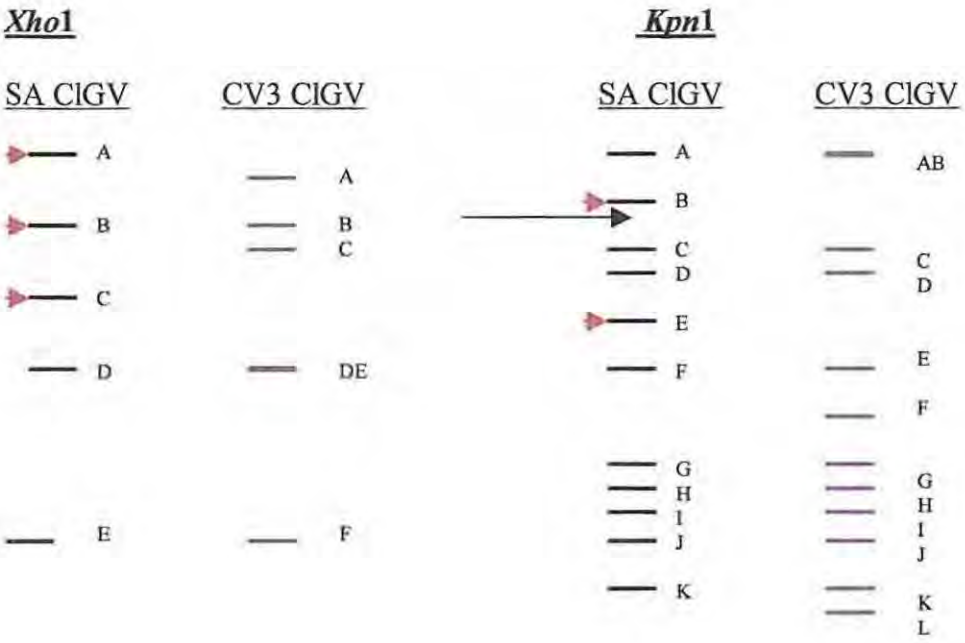


Figure 17: Electrophoresis on a 1 % agarose gel of ClGV-SA DNA and ClGV-CV3 DNA after digestion with restriction endonucleases. A 1 kb ladder was used as a molecular weight marker.

Figure 18: Diagram of ClGV DNA restriction fragment profiles produced as described in Fig. 17. This diagram is directly comparable to Fig. 17 with arrows indicating fragments that are present in the ClGV-SA digests but not in the ClGV-CV3 digests. Distances are not to scale.





For the *Eco*R1 digest, there were many fragments that were present in the digests of the ClGV-SA isolate that were absent in the ClGV-CV3 isolate. These include fragments A, B, D, F, K, M, O, R, S, T, U &V (Table 4). However there were too many fragments to represent diagrammatically.

3.2.3) Discussion

3.2.3.1) Single enzyme digests of ClGV DNA

ClGV DNA was initially digested with 10 restriction enzymes to produce RE profiles. Five enzymes namely *Bam*H1, *Eco*R1, *Kpn*1, *Sac*1 and *Xho*1, were selected to be used for the construction of physical maps. It was not possible to resolve all the fragments on single gel, therefore the best resolution of fragments larger than 3000 bp was obtained on a 0.7 % gel whereas fragments less than 2500 bp are most clear on a 1 % gel (Farrell, 1993). The digests using these five enzymes were therefore electrophoresed on 1 and 0.7 % gels. This was necessary so that all their fragments could be seen, for the calculation of the genome size (Figs. 13 & 14).

In the *Xho*1 digest a submolar band was present (Fig. 17), indicating the presence of genotypic heterogeneity. No other submolar bands were present in any of the other digests. Smith & Crook (1988) suggest that genotypic heterogeneity within a baculoviral population is typically indicated by the presence of submolar bands in RE profiles and it appears to be widespread among field isolates of granuloviruses. Most

baculovirses are a mixture of genotypes but it would be desirable to work with a cloned strain to eliminate variability (Crook *et al*, 1997). The digests were done several times to optimise conditions. The gel photographs in Figs. 13 & 14 showed a good resolution of all the ClGV fragments. The molecular weight markers run on all gels enabled the calculation of each fragments size (discussed later in the chapter). Each fragment was assigned a letter alphabetically, with A the largest fragment (presented later in the chapter).

The enzyme *Eco*R1 posed a problem since it was unable to restrict the DNA reproducibly, possibly due to a contaminant present in the DNA that inhibited the restriction enzyme. The DNA was subjected to another purification step using the Genomic tips (Qiagen). The enzyme was then able to successfully restrict the ClGV DNA. The sizes of some of the large fragments could not be estimated accurately and the intensity of some bands indicated that there might be two fragments of similar size present, therefore it was decided that double digests would be done.

3.2.3.2) Double digests of ClGV DNA

To extend the restriction enzyme data the ClGV DNA was digested with a combination of enzymes. This information would be useful for the construction of physical maps for each enzyme (using the single and double digest information) and for the calculation of genome size (since large fragments would be digested into smaller ones).

Four pairs of restriction enzymes were used to restrict the ClGV DNA. The profiles produced are presented in Figs. 15 & 16. Each fragment was lettered alphabetically (in lower case) and the size of each of these fragments was calculated. For a circular genome the number of fragments generated in the double digests must be equal to the total number of fragments present in both single digests of the restriction enzymes used. The summation of the molecular weights of fragments produced from the digests gives a total genome size (Gettig & MaCarthy, 1982). The calculation of the size of the ClGV genome using this data will be presented later in this chapter.

3.2.3.3) The comparison of RE profiles - ClGV-SA and ClGV-CV3

Both isolates of ClGV showed their own distinctive restriction enzyme profiles. The differences in their profiles are due to the addition or loss of restriction sites or the insertion or deletion of DNA (Goto *et al*, 1992), e.g. a single fragment can result from the loss of the restriction site between two adjacent fragments, or two fragments can result from the acquisition of a site in a single fragment. These types of changes are common in GV variants (Smith & Crook, 1988). Similarities between ClGV-SA and ClGV-CV3 were noted by the presence of identically sized fragments in their digests. However for each enzyme, their profiles differed in the number and position of restriction enzyme sites. For example, the largest fragment generated by an *Eco*R1 digest of ClGV-CV3 is 17.0 kbp, but this fragment was absent in the ClGV-SA profile due to a site being lost on the genome (Fig. 17). Other differences included the presence of 4 fragments in the *Sac*1 digest of ClGV-SA while the ClGV-CV3 digest only had 3 fragments (Fig. 17). The *Bam*H1 digest of ClGV-SA had 9 fragments as opposed to the 11 fragments in the ClGV-CV3 digest with *Bam*H1 (Fig. 17). In the *Kpn*1 profile, fragments sized 22.2 kbp and 11.8 kbp were present in the ClGV-SA profile but absent in the ClGV-CV3 profile (Fig. 17).

The differences between ClGV-SA and ClGV-CV3 are further highlighted in the construction of the physical maps (discussed in Chapter 4). The literature suggests that relatedness between isolates correlates to geographical origin. These results illustrate that the isolate of ClGV found in South Africa is distinct from the isolate found in the Cape Verde islands.

3.3) THE CALCULATION OF THE ClGV-SA GENOME SIZE

The size of a genome can be estimated by a summation of the sizes of the fragments generated by each restriction endonuclease (Goto *et al*, 1992). The sizes of all the fragments from both the single and double digests were calculated by comparison with the sizes of the molecular weight markers using the computer program called Program Gel (written by John R Thompson). Sizes of large fragments were estimated by double digests (Jehle & Fritsch, 1992).

3.3.1) Materials and methods

Using the profiles generated from both the single and double restriction enzyme digests (Figs. 13-16), the size of the ClGV genome was estimated. The total size was determined by adding the sizes of each individual fragment from a particular digest. A common size in kbp had to be determined for all digests.

3.3.2) Results

Three different molecular weight markers were run on all the agarose gels to assist in calculation of fragment sizes. They were λ*Bst*E II (for small fragments), λ*Hind* III (large fragments) and λ Mix 19 (very large fragments). The sizes of each of these marker fragments are presented in Table 6. The sizes determined for all fragments (both single and double digests) are presented in Tables 4 & 5.

Table 4: Estimated sizes in kbp of restriction fragments of singly digested ClGV-SA DNA. Fragments are labelled in order of decreasing size, **A** being the largest fragment.

FRAGMENT	*Eco*R1	*Bam*H1	*Kpn*1	*Sac*1	*Xho*1
A	9.97	32.00	27.00	33.0	57.50
B	8.78	24.95	22.20	30.4	23.50
C	8.55	11.50	16.20	27.5	14.50
D	7.76	9.85	15.00	22.0	12.10
E	7.60	9.70	11.83		4.65
F	7.58	7,93	10.00		
G	7.30	7.50	3.30		
H	5.35	6.10	2.30		
I	5.0	2.60	1.90		
J	5.0		1.65		
K	4.68		1.10		
L	4.53				
M	4.32				
N	4.0				
O	3.94				
P	3.70				

Q	2.85				
R	2.66				
S	2.39				
T	2.13				
U	1.65				
V	0.92				
W	0.85				
X	0.53				
TOTAL	**112.04**	**112.13**	**112.48**	**112.90**	**112.25**

Table 5: Estimated sizes in kbp of restriction fragments of double digested ClGV-SA DNA. Fragments are labelled in order of decreasing size, with **a** the largest fragment.

FRAGMENT	*Kpn*1 + *Sac*1	*Bam*H1+*Xho*1	*Bam*H1+*Sac*1	*Sac*1+*Xho*1
a	22.0	23.50	22.0	30.4
b	16.20	12.12	15.20	19.28
c	15.00	11.50	12.20	16.21
d	11.80	9.85	11.52	12.10
e	10.05	9.70	9.85	9.11
f	9.40	8.05	9.70	8.13
g	7.76	7.90	7.90	6.48
h	7.58	7.50	7.50	4.65
i	3.30	6.00	4.65	3.70
j	3.00	4.65	4.0	2.82
k	2.23	3.40	3.20	
l	1.98	3.13	3.0	
m	1.65	3.05	2.60	
n	0.997	2.60		
o	0.630			
TOTAL	**113.52**	**112.95**	**113.35**	**112.88**

Table 6: Sizes of the fragments (kbp) of the molecular weight markers used to calculate the sizes of the fragments generated from the restriction enzyme digests of ClGV DNA.

FRAGMENTS	λ Mix 19	λ*Hind* III	λ*Bst*E II
1	23.894	23.130	8.454
2	18.397	9.416	5.686
3	17.053	6.557	4.832
4	15.004	4.361	4.324
5	12.220	2.322	3.678
6	10.086	2.10	2.322
7	8.814		1.929
8	8.217		1.371
9			1.264

3.3.3) Discussion

Initially standard curves were drawn to calculate genome size, but they proved to be very inaccurate. The sizes of the large fragments could not be accurately determined until marker λ Mix 19 was used on the agarose gels. This marker has fragments of very large sizes, which enabled the sizes of the large ClGV fragments to be determined. Program Gel determined sizes using the molecular weights of the marker fragments, their distances migrated and the migration distances of the ClGV fragments. The total genome size was calculated by adding together individual fragment sizes in each digest and a figure of 112 kbp was calculated for most digests. Thus, the size of the ClGV-SA genome is the same as that of the ClGV-CV3 genome (estimated to be 112.4 kbp by Jehle & Fritsch, 1992).

3.4) PLASMID LIBRARY OF THE ClGV-SA GENOME

A restriction fragment library representing 91 % of the ClGV genome was constructed. *Eco*R1 and *Bam*H1 fragments were cloned into pBluescript II SK (+) plasmid vector. Cloning was carried out with digests of genomic DNA that had been cut with these enzymes or with individual bands that had been cut from gels in the case of the larger fragments. The cloned inserts were identified by their

electrophoretic mobility and by Southern blot hybridisations. Hybridisations of labelled cloned plasmids to Southern blots of viral DNA fragments can confirm the viral origin of the inserts (Goto *et al*, 1992). The construction in plasmids of a restriction fragment library of the ClGV-SA genome and the construction of physical maps for several restriction endonucleases are essential for further experiments. The library should ideally overlap and cover the entire genome (Crook *et al*, 1997). For most purposes it is useful to have a set of clones that cover most of the genome. These libraries are useful to use for the detection and location of essential genes in the genome, e.g. the *egt* and granulin genes. Crook *et al* (1993) used such a library to locate the *iap* gene in the CpGV genome. Plasmid DNAs were used as probes and hybridised to viral DNA fragments that had been digested with the same restriction enzymes used for cloning and then electrophoresed on agarose gels. The separated DNA fragments were Southern transferred to nitocellulose filters and hybridised with probes of cloned plasmid DNA labelled with a radioactive or non-radioactive label.

3.4.1) Materials and methods

A library of ClGV-SA DNA fragments was constructed by ligating restricted viral DNA fragments into pBluescript SK (+) plasmid vector (Appendix 2) using T4 DNA ligase (Roche). Recombinant plasmids were cloned and propagated in *E.coli* DH5α cells and purified by alkaline lysis (Sambrook *et al*, 1989).

DIGESTION OF ClGV DNA BY *Eco*R1 and *Bam*H1

The digest was set up as follows:

x μl DNA (800 ng)
2 μl *Eco*R1 / *Bam*H1 (20 units)
2 μl Buffer H /B (10 x)
x μl ddd water
20 μl

The digest was incubated at 37°C for 2 hours, and then electrophoresed on a 0.7 % TAE agarose gel to confirm digestion of the ClGV DNA.

PREPARATION OF pBLUESCRIPT SK (+) VECTOR FOR LIGATION

The vector was linearsised with enzyme as follows:

7 μl Vector (pBSK+) (5 μg) (Stratagene)
1 μl *Eco*R1 / *Bam*H1 (20 units)
10 μl 10 x Buffer H/B
82 μl ddd water
100 μl

The digest was incubated at 37°C for 2 hours. The enzymes were inactivated by incubating the digest at 65°C for 15 minutes, which was electrophoresed on a 1 % gel (100 V for 1 hour) to confirm that the plasmid had linearised. Once linear, the plasmid was dephosphorylated.

DEPHOSPHORYLATION OF VECTOR

This was performed as follows: 45 μl vector (pBSK+) (5μg) (Stratagene)
5 μl Shrimp alkaline phosphatase (Roche) (5U)
1 μl 10 x dephosphorylation buffer

The mixture was incubated at 37°C for 1 hour. The enzyme was then inactivated by incubating the reaction at 65°C for 15 minutes.

LIGATION OF *Eco*R1 FRAGMENTS INTO PLASMID VECTOR

Using Roche reagents, the ligation reaction was set up as follows:

1 μl ATP (10 mM)
1 μl pBSK+ vector (dephosphorylated) (Stratagene)
6 μl Insert DNA (ClGV digested with *Eco*R1/*Bam*H1)
0.5 μl T4 DNA ligase (1 U) (Roche)
1 μl 10 x ligation buffer (Roche)
9.5 μl

The ligation was incubated at 4°C overnight. One of the controls for the ligation experiment did not contain ClGV DNA, and the second control used vector that had not been dephosphorylated.

TRANSFORMATION OF RECOMBINANTS INTO *E.coli* CELLS

The ligation mix was transformed into *E.coli* DH5α cells using electroporation or heat shock. Refer to Appendix 2 for the protocols used. Transformants were grown up overnight at 37°C on LB/AMP plates (treated with IPTG and X-gal) and were then

screened for white/blue colonies. When a foreign gene is inserted into the pBSK+, insertional inactivation of the *lacZ* gene occurs, resulting in the growth of white colonies. White colonies containing insert DNA in the plasmid were then subcultured for plasmid purification.

PLASMID PURIFICATION

White colonies were subcultured overnight in 5 ml of LB/AMP broth at 37°C. Firstly, Smart Preps were preformed (refer to Appendix 2 for the method used) to investigate whether the desired clones were present. If the clone contained an insert, it was subcultured overnight in 5 ml of LB/AMP broth at 37°C and alkaline lysis was performed for plasmid DNA isolation. Refer to Appendix 2 for the protocol. After plasmid DNA was isolated it was digested with either *Eco*R1 or *Bam*H1 for 2 hours at 37°C and these digests were electrophoresed on a 1 % agarose gel.

SOUTHERN BLOT ANALYSIS

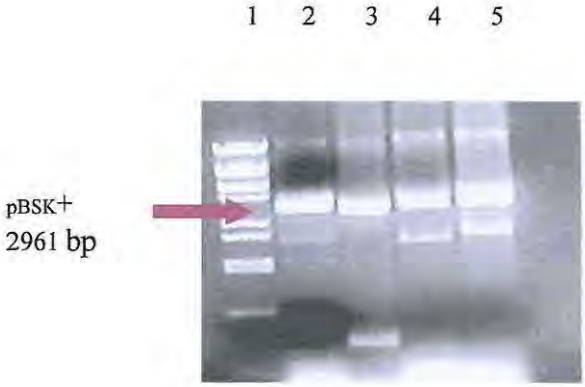
To verify if a certain insert had been cloned, the clone was labelled non-radioactively with DIG (Roche). It was then used to probe a Southern blot of all the RE digests of ClGV DNA. The protocols used will be discussed in the next chapter. The detection occurred on X-ray film via chemiluminescence. The probes bound to particular fragments in each RE digest. One lane of each Southern blot contained ClGV-SA digested with *Eco*R1 to enable identification of the labelled *Eco*R1 fragment used as a probe.

3.4.2) Results

The plasmid vectors were successfully linearised with both enzymes (Fig. 19). *Eco*R1 and *Bam*H1 digests of ClGV were then "shot gun cloned" into the vector, some of which are presented in Figs. 20-22. The size of each fragment cloned was determined using the molecular weight markers (Table 6) and Program Gel (as done in 3.3) to determine which fragments had been cloned. A stock was made of each clone in glycerol/LB media and they were stored at –70°C. Streak plates of each clone were also made on LB/AMP medium and stored at 4°C.

1 – 1kb ladder marker
2 – undigested pBSK+ (2961 bp)
3 – pBSK+ digested with *Bam*H1
4 – pBSK+ digested with *Eco*R1

Figure 19: Linearised plasmid pBluescript (SK+)



1 – λ/*Bst*E II marker
2 - *Eco*R1 T in pBSK+
3 – *Eco*R1 X in pBSK+
4 – *Eco*R1 T in pBSK+
5 – *Eco*R1 S in pbSK+

Figure 20: Cloning of E*co*R1 fragments S, T & X into plasmid vector pBSK+.

1 – λ/*Bst*E II marker
2 - *Eco*R1 J in pBSK+
3 – *Eco*R1 B in pBSK+
4 – *Eco*R1 K in pBSK+
5 – *Eco*R1 in pBSK+
7 - *Eco*R1 O in pBSK+
9 – *Eco*R1 N in pBSK+
10 – *Eco*R1 R in pBSK+
12 – *Eco*R1 G in pBSK+

Figure 21: Cloning of E*co*R1 fragments into plasmid vector pBSK+

1 – λ/*Bst*E II marker
2 - *Bam*H1 G in BSK+
3 – *Bam*H1 F in BSK+

Figure 22: Cloning of *Bam*H1 fragments G & F into plasmid vector pBSK+

Southern blots of ClGV-SA DNA cut with various restriction enzymes were probed with labelled *Eco*R1 fragments. Detection was done on X-ray film as seen in Fig. 23 which illustrates the restriction digests of ClGV-SA and ClGV-CV3 probed with labelled *Eco*R1 fragment S.

1 - *Bam*H1/ SA digest
2 - *Bam*H1/CV3 digest
3 – *Eco*R1/SA digest
4 – *Eco*R1/CV3 digest
5 – *Kpn*1/SA digest
6 – *Kpn*1/CV3 digest
7 – *Sac*1/SA digest
8 – *Sac*1/CV3 digest
9 – *Xho*1/SA digest
10 – *Xho*1/CV3 digest

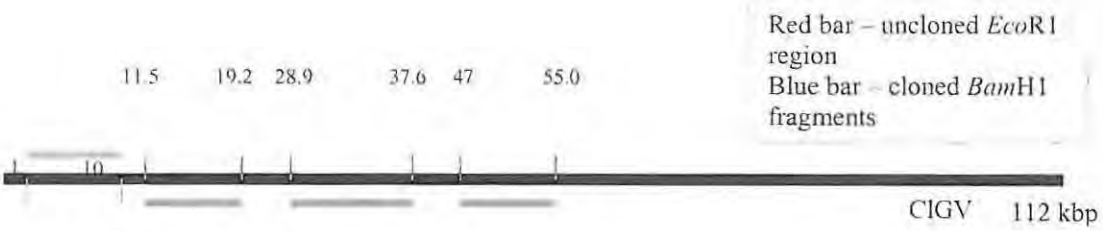
Figure 23: Southern Blot of RE digests of ClGV-SA DNA probed with labelled *Eco*R1 S. The signal indicates the RE fragment in each digest containing the sequences homologous to fragment S. The identity of labelled fragment S was confirmed since the probe bound to fragment S in the *Eco*R1 digest (Fig. 20). Refer to Table 9 for the *Eco*R1 probes that were used.

Table 7 lists all the fragments from the *Eco*R1 and *Bam*H1 digests that were successfully cloned into the plasmid vector. Cloned fragments are indicated by an (*).

Table 7: Fragments of ClGV-SA digested with *Eco*R1 and *Bam*H1 that have been cloned into plasmid vector pBluescript SK (+).

FRAGMENT	*Eco*R1 (kbp)	*Bam*H1 (kbp)
A	9.97	32.00
B	8.73*	24.95
C	8.55*	11.50
D	7.76*	9.95
E	7.60*	9.70
F	7.58*	7,93*
G	7.30*	7.50*
H	5.35*	6.10*
I	5.0*	2.60*
J	5.0*	
K	4.65*	
L	4.53*	
M	4.32*	
N	4.0*	
O	3.94*	
P	3.70*	
Q	2.85*	
R	2.66*	
S	2.39*	
T	2.13*	
U	1.65*	
V	0.92*	
W	0.85*	
X	0.53*	
TOTAL	**112.04**	**112.13**

Figure 24: Diagram illustrating the different regions of the ClGV genome that have been cloned into plasmids for the construction of a plasmid library.



3.4.3) Discussion

91 % of the ClGV genome was cloned into plasmid vectors. The uncloned region extended from 1–10 kbp (Fig. 24). The reason for this region not being cloned is its large size and the lack of other restriction enzyme sites in this region. For this particular region, the smallest *Eco*R1 fragment was 10 kbp, which could not be successfully cloned into pBluescript despite numerous attempts. 23 out of the 24 *Eco*R1 fragments and 4 out of the 9 *Bam*H1 fragments were cloned, producing a plasmid library that did overlap in certain regions. The remaining *Bam*H1 fragments could not be cloned due to their large sizes. The other enzymes used also produced many fragments of large sizes, which were not suitable for constructing a library.

The cloned fragments were authenticated by determining their sizes. This was achieved by calculating the fragment size using Program Gel, which used the sizes of the molecular weight markers, their migration distances and the migration distances of the fragments on the 1 % agarose gels. Probing of the Southern blots of the RE digests of ClGV DNA with certain cloned fragments as probes, enabled the location of these fragments to be determined in the ClGV genome as well as verifying which fragment had been labelled (Fig. 23). This technique therefore had an advantage since the physical maps for these enzymes could be constructed simultaneously.

The construction of a plasmid library of the genome will be useful since the library can be used to find the position of essential genes in the genome, e.g. granulin (Chapter 5). Another benefit of this library is for sequencing purposes. Sequencing would provide the basis for identification of genes involved in the pathology of the virus (Hashimoto *et al*, 2000). If any particular region of the genome needs to be sequenced, the fragment is already cloned in a vector, eliminating the cloning step in the sequencing process.

CHAPTER FOUR: PHYSICAL MAPS OF THE ClGV GENOME

4.1) INTRODUCTION

Detailed physical maps of viral genomes are required as a basis for in depth studies on the molecular biology of the virus (Crook *et al*, 1997). Physical maps are largely determined by reciprocal digests of viral DNA using restriction endonucleases and using Southern blot analysis of cloned or genomic viral DNA fragments (Hashimoto *et al*, 1996). These maps can be used to compare different isolates of virus to determine the relatedness of the viruses.

Mapping of restriction enzyme sites in the genome is done by digesting the DNA with single enzymes followed by digesting with a second restriction endonuclease. It is not necessary to carry out double digests with all possible combinations of enzymes. These procedures allow the common end fragments in the reciprocal double digest to be identified together with the secondary enzyme site present in a particular primary digested fragment (Possee & Kelly, 1988). The constructions of maps are also facilitated by using hybridisations to align RE digest products (Hashimoto *et al*, 1996). Mapping is also greatly aided by the construction of plasmid libraries of the genome (Crook *et al*, 1997). In instances where ambiguity remains certain fragments can be cloned into plasmid vectors. Cloned plasmid DNAs are used as probes in hybridisation experiments where the position of a fragment in the genome is largely determined by the location that the probe binds to in a Southern blot of the viral DNA (Crook *et al*, 1997).

Physical maps are generally linearised for the selected restriction endonucleases. Each cleavage site on the genome is indicated by a vertical line and as a percentage of the genome. A zero point of the map is chosen. Positions of restriction enzyme sites on the map are shown in map units. Using single and double digest information and hybridisation data, complete physical maps for ClGV-SA were constructed for *Bam*H1, *Eco*R1, *Kpn*1, *Sac*1 and *Xho*1.

4.2) MATERIALS AND METHODS

ClGV-SA DNA was digested with *Bam*H1, *Eco*R1, *Kpn*1, *Sac*1 and *Xho*1 singly and in combinations. Maps for *Bam*H1, *Xho*1, *Kpn*1 & *Sac*1 were constructed first due to the ease of interpreting fewer fragments and a map for *Eco*R1 was constructed later. Restriction enzyme digests were performed as in Chapter 3 and these digests were electrophoresed alongside one another on 0.7 % and 1 % agarose gels. The positions of fragments could not be determined without ambiguity. Probes of these fragments were made and their positions on the genome were determined using Southern blotting analysis. In the Southern blots, ClGV DNA from both the SA and the CV3 isolates were restricted with *Bam*H1, *Eco*R1, *Xho*1, *Kpn*1 & *Sac*1 and transferred onto a nitrocellulose filter.

4.2.1) Southern blotting of restriction enzyme fragments

Capillary transfer transferred digested ClGV-SA and ClGV-CV3 DNA from an agarose gel to nitrocellulose membranes. The gel was soaked in depurination solution for 10 minutes with shaking (refer to Appendix 3 for solutions used). The gel was then rinsed in distilled water, followed by a soak in denaturation solution for half an hour with gentle shaking. The gel was rinsed again in distilled water and soaked twice in neutralisation solution for 15 minutes on a rocker. For the transfer to nitrocellulose membrane, 6 pieces of Whatmann paper (3 MM) were cut out with three pieces larger than the size of the gel and three pieces the same size as the gel. The larger filters were soaked briefly in 20 x SSC buffer. To set up the transfer, cling film was placed on the bench top with the soaked large filters placed on top. The gel was then placed on top of the filters with a piece of nitrocellulose (the same size as the gel) placed on top of the gel. The cling film was wrapped around the edges of the gel. The three smaller pieces of Whatmann paper were then placed on the nitrocellulose followed by a wad of paper towel and a weight. This arrangement was left overnight to allow the transfer of the DNA to the nitrocellulose to occur. The next day the apparatus was disassembled and the membrane was allowed to dry at room temperature. The gel was soaked in ethidium bromide (10 mg/ml) for 30 minutes and then photographed to ensure that all digested fragments had been transferred to the nitrocellulose. Once dry the DNA was fixed onto the nitrocellulose membrane by exposure to UV light for 5 minutes on the transilluminator. The membrane was stored in cling film at 4°C till use.

4.2.2) Construction of labelled probes using the DIG labelling and detection system

A non-radioactive label digoxigenin (DIG) was used for the labelling of fragments (DIG High Prime DNA Labelling and Detection Starter Kit II, Roche). DIG labelled DNA probes were generated by the random priming (Sambrook *et al*, 1989).

4.2.2.1) Labelling of probes

The DIG High Prime mix contained digoxigenin-dUTP, Klenow enzyme and the buffer reagents. DNA fragments of at least 100 bp as well as linearised plasmid DNA could be labelled. For optimal results the template DNA had to be linearised and for Southern blotting the template DNA had to be separated from the vector by agarose gel electrophoresis. The protocol was designed for labelling of 3 to10 μg of DNA and volumes were scaled up for greater concentrations of DNA. The determination of labelling efficiency was done by a direct detection method, which was explained in the instruction manual (DIG instruction manual, 2001).

Clones from the plasmid library or restriction fragments cut out of gels were used as probes. Fragments were excised from agarose gels using an extraction kit (Nagel – Macherey, Appendix 3). DNA was extracted from the fragments and then precipitated using two volumes of 95 % ethanol and 1/10th volume 3 M sodium acetate. The DNA was incubated at –20°C for 20 minutes to overnight and then centrifuged at 13 000 g for 20 minutes. The DNA pellet was washed with 70 % ethanol and then allowed to dry. The DNA was finally resuspended in triple distilled water (16 μl) and was ready for DIG labelling. The concentration of the probe to be labelled was determined by making a 1/100 dilution of the probe and calculating its concentrations using a Genequant (Pharamacia Biotech). A sufficient concentration of 0.5 μg/ml was required for labelling. The probe was then boiled for 10 minutes and immediately cooled on ice for 2 minutes. 4 μl of DIG High Prime Mix was added to the probe and labelling occurred overnight at 37°C for 20 hours. DIG labelling was ended by adding 2 μl of 0.2 M EDTA (refer to Appendix 3 for solutions made). The probe was stored at –20°C until use.

4.2.2.2) Prehybridisation and hybridisation

Once the probe had been labelled, the blot to be probed was prepared for hybridisation. The hybridisation temperature was calculated, using a formula presented in the DIG instruction manual (page 16), to be 38°C for ClGV-SA DNA. 20 ml of hybridisation buffer (DIG Easy Hyb) was prewarmed to 38°C. The blot was placed in the hybridisation bottle with 10 ml of the prehybridisation solution and incubated in the hybridisation oven for 1 hour. The probe was boiled for 5 minutes and cooled on ice for 2 minutes. It was then added to the remaining 10 ml of hybridisation solution. The prehybridisation solution was poured off and the hybridisation buffer with probe was added. This was incubated overnight at 38°C, with agitation.

4.2.2.3) Washes

The blot was washed twice in ample 2 x SSC, 0.1 % SDS at room temperature with agitation for 5 minutes. Thereafter the blot was washed twice for 15 minutes in 0.5 x SSC, 0.1 % SDS at 65°C with agitation (in the hybridisation oven). The 0.5 x SSC solution had to be prewarmed to 65°C overnight.

4.2.2.4) Immunological detection

The hybridised probes were immunodetected with an alkaline phosphatase-labelled anti-digoxigenin and then visualised with chemiluminescence substrate, CSPD. The enzymatic dephosphorylation of CSPD by alkaline phosphatase led to light emmision which was recorded on X-ray film (DIG instruction manual, 2001).

All incubations were performed at room temperature with agitation. The blot was washed for 5 minutes in washing buffer with agitation. It was then incubated in 80 ml blocking solution (freshly made up) for 30 minutes at room temperature. Next the blot was incubated in 20 ml of freshly made anti-DIG solution for 30 minutes. The blot was then washed twice in 100 ml of washing buffer for 15 minutes. A five minute incubation in 20 ml Detection buffer followed. Thereafter the blot was transferred to a piece of cling film and 1 ml of ready-to–use CSPD reagent was added. The cling film was wrapped around the blot and it was allowed to incubate for 5 minutes. The excess CSPD was squeezed out and the blot was dried at 37°C for 10 minutes (to enhance the chemiluminescence). The blot was then placed into an autoradiography cassette and

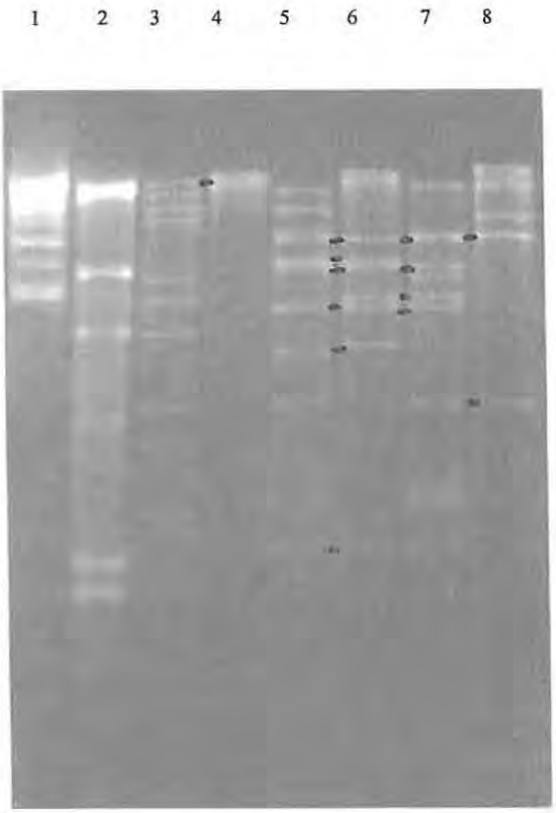
covered with X-ray film. This was incubated for 15-25 minutes depending on the concentration of the probe. After the incubation, the X-ray film was developed as follows: 1 minute in developer, 30 seconds in 2 % acetic acid and 3 minutes in fixer. The film was washed in water for 30 minutes and then hung up to dry. Refer to Appendix 3 for the solutions used.

4.2.2.5) Stripping of blots

To strip the blot for re-probing, it was washed in distilled water for 1 minute. Then it was incubated in 50 ml of stripping solution (Appendix 3) at 37°C twice for 15 minutes. After stripping the blot was stored in 2 x SSC buffer at 4°C (until probed again).

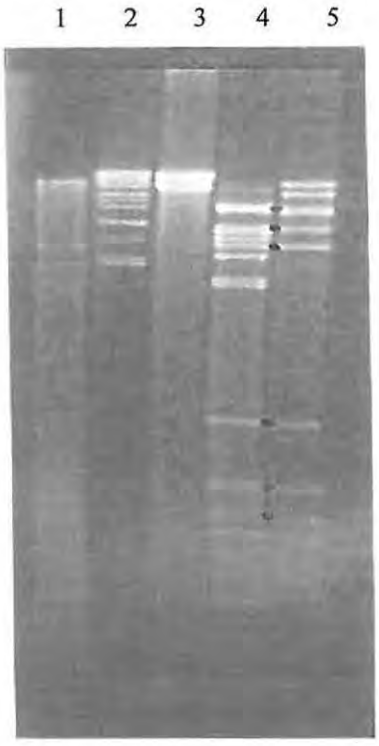
4.3) RESULTS

Preliminary physical maps for the five restriction enzymes selected were constructed using the single and double digest information presented in Figs. 13-16 in Chapter 3. The sizes of all the restriction enzyme fragments generated are presented in Tables 4 and 5 in Chapter 3. To enable direct comparisons between the single and double enzyme digests of ClGV DNA, RE digests were electrophoresed alongside one another on agarose gels. Fig. 25 presents two such gels where ClGV-SA DNA was digested with *Bam*H1, *Bam*H1+*Xho*1, *Xho*1, *Sac*1+*Xho*1, *Sac*1, *Sac*1+*Bam*H1 and *Sac*1, *Sac*1+*Kpn*1, *Kpn*1. These gels allowed direct comparisons to be made; to determine where new RE sites had been created and which RE sites remained.



1 – λ Mix 19 marker
2 – λ/*Hind* III marker
3 – *Sac*1/ - *Xho*1 digest
4- *Sac*1 digest
5- *Sac*1/ *Bam*H1 digest
6- *Bam*H1 digest
7- *Xho*1/ *Bam*H1 digest
8- *Xho*1 digest

(A)



1 – λ/*Hind* III marker
2 - λ Mix 19 marker
3 - *Sac*1 digest
4 – *Sac*1/*Kpn*1 digest
5 – *Kpn*1 digest

(B)

Figure 25 (A) & (B): Restriction enzyme profiles of single and double digests of ClGV-SA DNA electrophoresed on 0.7 % agarose gels with λ/*Hind* III and λ Mix 19 used as molecular weight standards. Dots indicate bands occurring in both the single and double digests.

Using this data, mapping of fragments in a circular genome was done using the two assumptions:

1) Fragments from a single digest which were still present after a double digest, did not contain a restriction site for the secondary enzyme.

2) Given that DNA was circular, the total number of fragments produced from two single digests had to equal to the number of fragments from the equivalent double digests.

Tables were constructed using the single and double enzyme digest information and physical maps were drawn using this data (Appendix 3).

Preliminary physical maps were drawn using the digest information, but the positions of certain fragments in the genome remained ambiguous. These fragments were labelled with DIG and used to probe Southern blots of the restricted ClGV DNA. The positions of these fragments in the ClGV-SA genome were largely determined by identifying the fragments to which the probe hybridised in Southern blotting of the ClGV-CV3 genome, for which Jehle & Fritsch (1992) had constructed physical maps for the same restriction enzymes. If a probe hybridised to a particular fragment in the ClGV-CV3 genome, then that fragment was placed in the same position in the ClGV-SA genome. Of the 22 blots performed two examples are presented in Figs. 26 and 27. In Fig. 26 the position of fragment A of ClGV-SA/*Bam*H1 in the genome was investigated. The probe (DIG labelled *Bam*H1 fragment A) hybridised to *Bam*H1 A, *Xho*1 D, *Xho*1 E and *Sac*1 D in the ClGV-SA genome. In the ClGV-CV3 digest by *Bam*H1, the probe hybridised to fragment A, which suggested that *Bam*H1 A from the SA isolate lay at the same map position as *Bam*H1 A in the CV3 isolate. In Fig. 27 the position of fragment A of ClGV-SA/*Kpn* was investigated. The results showed that the fragment hybridised to *Bam*H1 C, *Sac*1 D, *Xho*1 A and *Kpn*1 A in the ClGV-SA genome. The fragment also hybridised to *Kpn*1 B in ClGV-CV3 digest suggesting that this fragment lay at the same map position as ClGV-CV3 *Kpn*1 B. Table 8 presents the hybridisation data obtained from the probing using different labelled fragments. Using all the above information, physical maps were constructed for the restriction enzymes *Bam*H1, *Kpn*1, *Xho*1 and *Sac*1 (Fig. 28).

Table 8: ClGV-SA fragments labelled as probes that hybridised to restriction fragments of ClGV-SA and ClGV-CV3

PROBES	**SA**	**CV3**	**SA**	**CV3**	**SA**	**CV3**	**SA**	**CV3**	**SA**	**CV3**
	***Bam*H1**	***Bam*H1**	**E*co*R1**	***Eco*R1**	***Kpn*1**	***Kpn*1**	***Sac*1**	***Sac*1**	***Xho*1**	***Xho*1**
*Kpn*1 E	C	-	A, (B)	A	E	A	-	-	A	C, B
*Xho*1 B	B	A	C, D, E	-	D	B	-	-	B	A
*Bam*H1D	D	-	E	-	B, C	-	C	C	A	-
*Bam*H1F	F	D	-	C	B, (A)	-	A	-	A	B, (C)
*Xho*1 C	A	-	B, C	-	A	B	-	-	C	-
*Kpn*1 B	B	B	C, D, E	B	B	C	C	C	B	A
*Bam*H1E	E	C	B, C	C	C	A	B	B	A	B
*Bam*H1B	B	B	-	-	A,F	E	A	A	B,C	A
*Sac*1 D	A	A	-	-	A	B	D	-	D	E
*Bam*H1 A	A	A	-	-	F	B,E	D	A	D	E
*Kpn*1 A	C	J,H	-	-	A	B	D	A	A	-

1 – SA/*Bam*H1 digest
2 -CV3/B*am*H1 digest
3 – SA/*Eco*R1 digest
4 – CV3/*Eco*R1 digest
5 – SA/*Kpn*1 digest
6 – CV3/*Kpn*1 digest
7 – SA/*Sac*1 digest
8 – CV3/*Sac*1 digest
9 – SA/*Xho*1 digest
10 – CV3/ *Xho*1 digest

Figure 26: Hybridisation of DIG labelled fragment A of ClGV-SA/*Bam*H1 in a Southern blot of restricted ClGV-SA and ClGV-CV3 DNA.

1 – SA/*Bam*H1 digest
2 -CV3/B*am*H1 digest
3 – SA/*Eco*R1 digest
4 – CV3/*Eco*R1 digest
5 – SA/*Kpn*1 digest
6 – CV3/*Kpn*1 digest
7 – SA/*Sac*1 digest
8 – CV3/*Sac*1 digest
9 – SA/*Xho*1 digest
10 – CV3/ *Xho*1 digest

Figure 27: Hybridisation of DIG labelled fragment A of ClGV-SA/*Kpn*1 in a Southern blot of restricted ClGV-SA and ClGV-CV3 DNA.

PHYSICAL MAPS OF THE ClGV-SA GENOME – legend overleaf

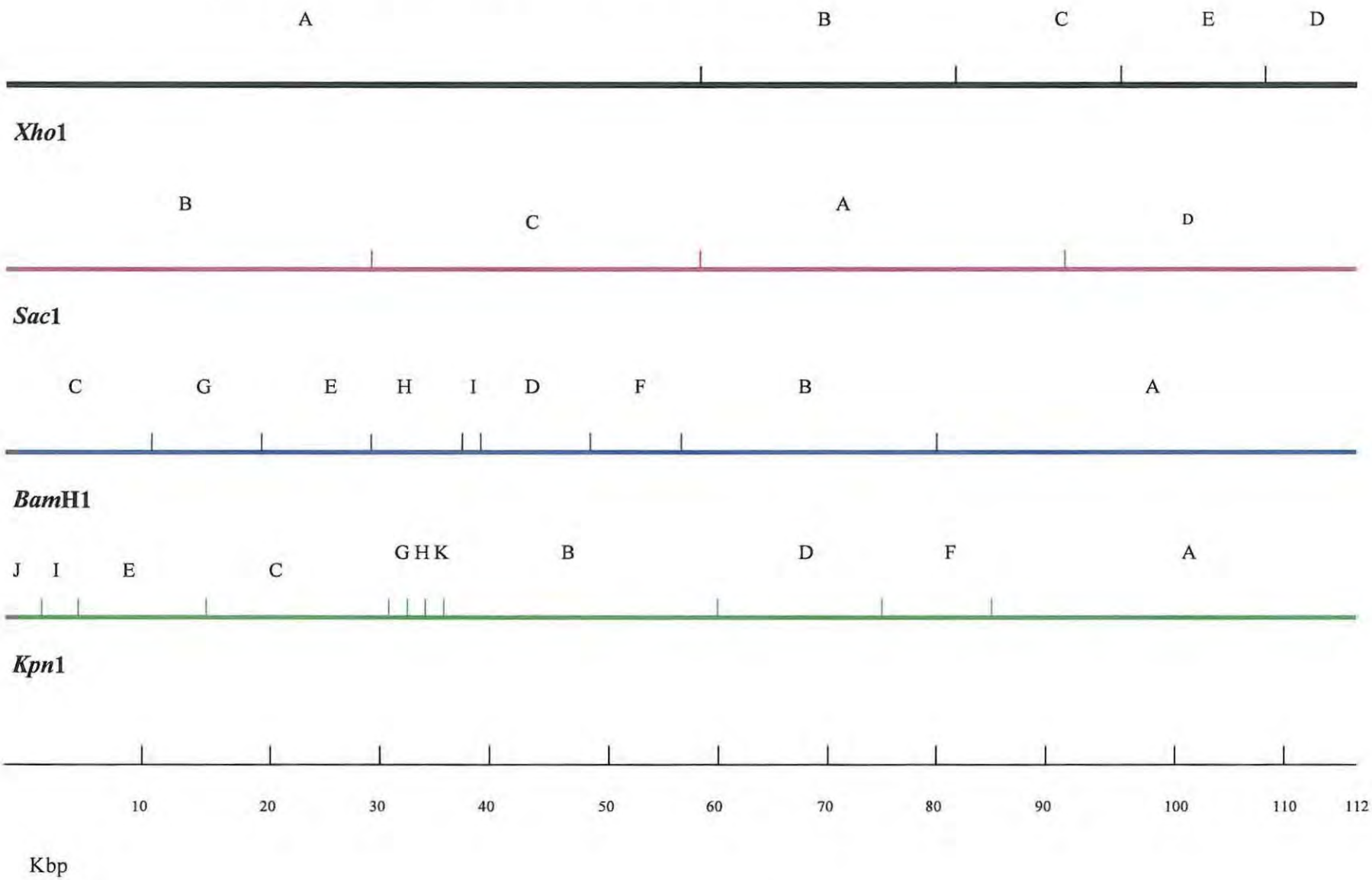


Figure 28: Physical maps of ClGV-SA DNA for *Bam*H1, *Kpn*1, *Xho*1 and *Sac*1. The circular genome was linearised. A vertical line indicated each cleavage site and the position of RE sites on the map are shown in map units.

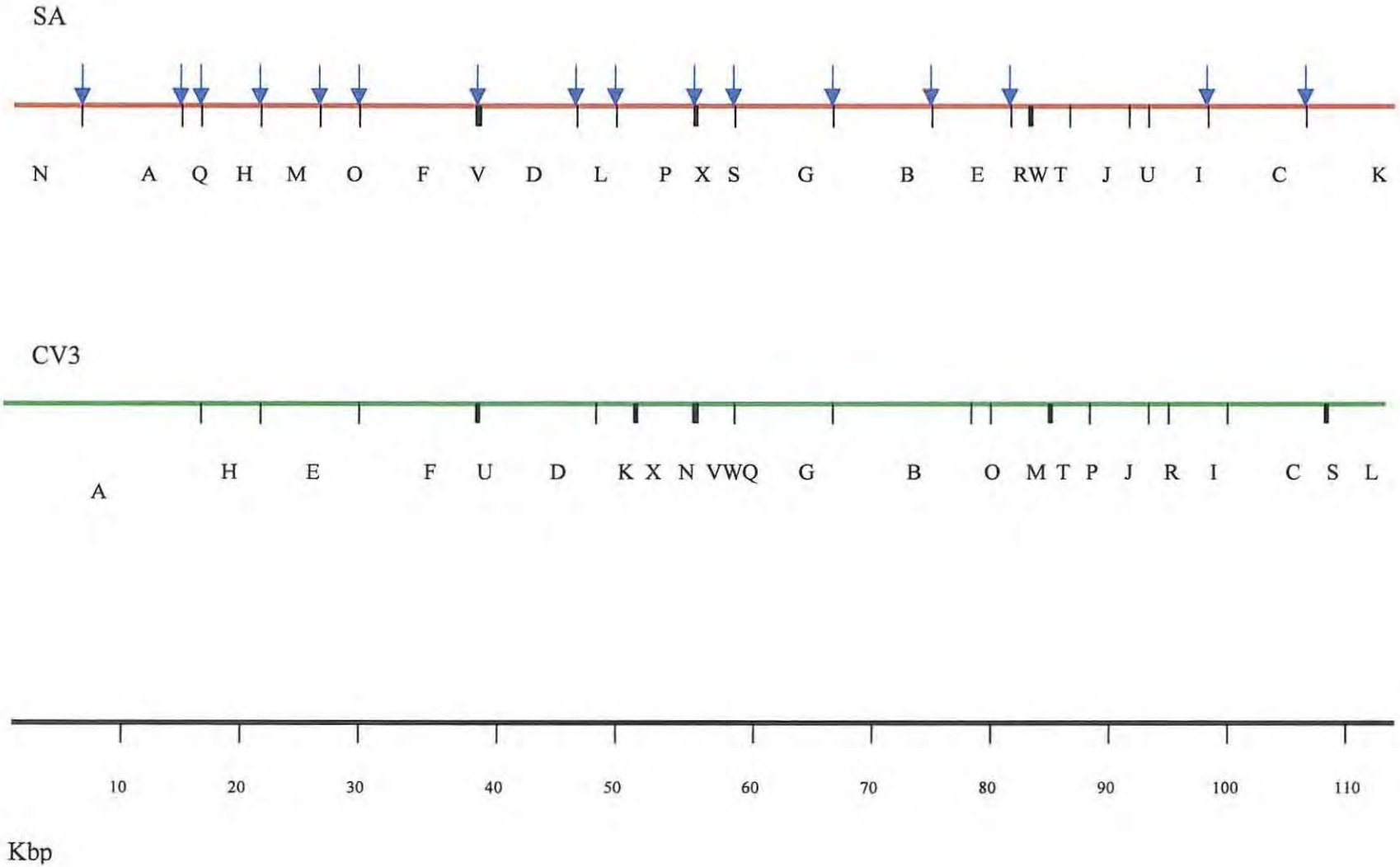
A more extensive map was constructed for ClGV-SA *Eco*R1 since there were 24 restriction enzyme fragments. Such a map would aid in the sequencing of the genome and in detailed comparative analyses of ClGV-SA to other baculoviruses (Wormleaton & Winstanely, 2001). Certain *Eco*R1 fragments were placed into position by virtue of being identical in size to the CV3 *Eco*R1 fragments. The remainder of the fragments were mapped by labelling them with DIG and using them to probe the Southern blots of restricted ClGV DNA. These fragments were obtained from the plasmid library of *Eco*R1 fragments (refer to Chapter 3). The fragments that hybridised to the probe were recorded and from this an estimate of the position of the fragment was made. The data obtained from these hybridisation experiments is presented in Table 9. Using this information a physical map for *Eco*R1 was constructed (Fig. 29).

The zero point of each map was assigned as the smallest fragment that contained the granulin gene. The position of the gene in the genome was located by labelling a fragment of the gene obtained by PCR with DIG and using it to probe the Southern blots (to be discussed in Chapter 5). The smallest fragment in each digest containing the gene was assigned to the zero point or first fragment in the linear map.

Table 9: Hybridisation data generated for the *Eco*R1 physical map

PROBES	SA	CV3	SA	CV3	SA	CV3	SA	CV3	SA	CV3
	*Bam*H1	*Bam*H1	*Eco*R1	*Eco*R1	*Kpn*1	*Kpn*1	*Sac*1	*Sac*1	*Xho*1	*Xho*1
*Eco*R1 S	A	A	S	P	D	-	-	A	E, C	A,F
*Eco*R1 V	-	-	V	-	C, (E)	A	B, C	-	A	B
*Eco*R1 P	-	-	P	N	B	C	C	C	A	B
*Eco*R1 M	E,G	F, C	M	H	-	A	B	B	A	C
*Eco*R1 A	C	J, H	A	A	C	A	-	-	A	-
*Eco*R1 D	-	-	D	-	B	-	A	-	A	-
*Eco*R1 B	B	B	B	B	D	D	-	B	C	A
*Eco*R1 Q	G, E	C	Q	A, H	E	A	B	B	A	B
*Eco*R1 E	A,B	E, A	E	B, O	D	D, E	-	-	B	A
*Eco*R1 O	C	H, F	O	E	E	A	B	B	A	C
*Eco*R1 R	A	A	R	O	-	-	A	A	B	A

Figure 29: *Eco*R1 PHYSICAL MAPS OF THE ClGV-SA & ClGV-CV3 GENOMES
Differences are indicated by arrows



The physical maps of the South African and the Cape Verde isolates were aligned for comparison (Fig. 30).

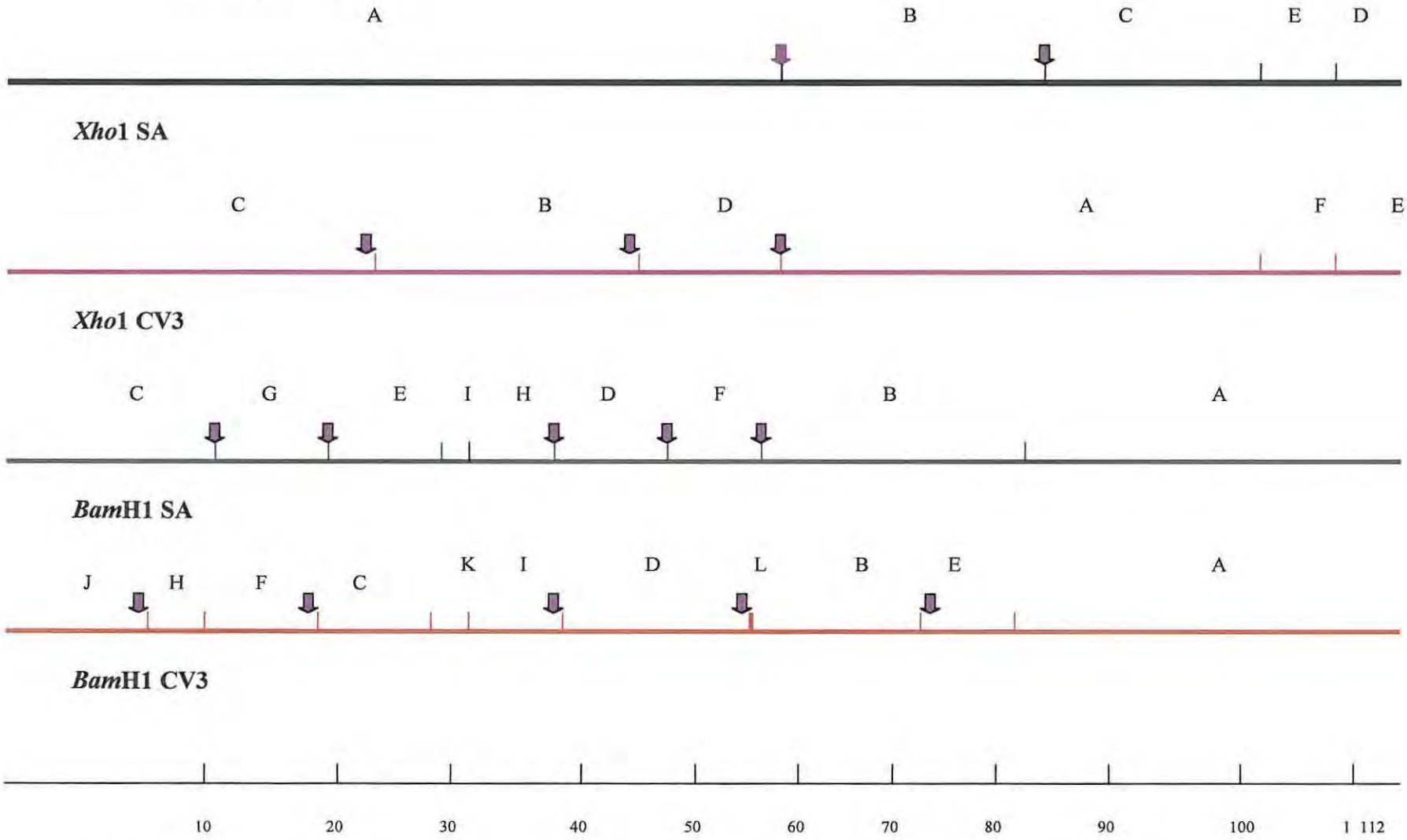
4.4) DISCUSSION

Using the information from the single and double digests of ClGV-SA preliminary maps were constructed for *Bam*H1, *Kpn*1, *Sac*1 and *Xho*1. By analysing the restriction enzyme profiles produced, certain deductions could be made. By comparing the *Xho*1, *Xho*1/*Bam*H1 and *Bam*H1 digests, it could be determined that fragment *Xho*1 B did not contain a *Bam*H1 site since the fragment was still present in the double digest of *Xho*1 with *Bam*H1.The same could be applied for fragment *Xho*1 E. However fragment *Xho*1 A did possess a *Bam*H1 site since this fragment was lost in the double digest with *Bam*H1. From the double digests it could be deduced that fragment *Xho*1 A was cleaved by *Bam*H1 into fragments c, d, e, g, h, m, n, l, and k in the double digest (Table 18 and Fig. 51). Using these principles, fragments from each restriction enzyme digest were mapped.

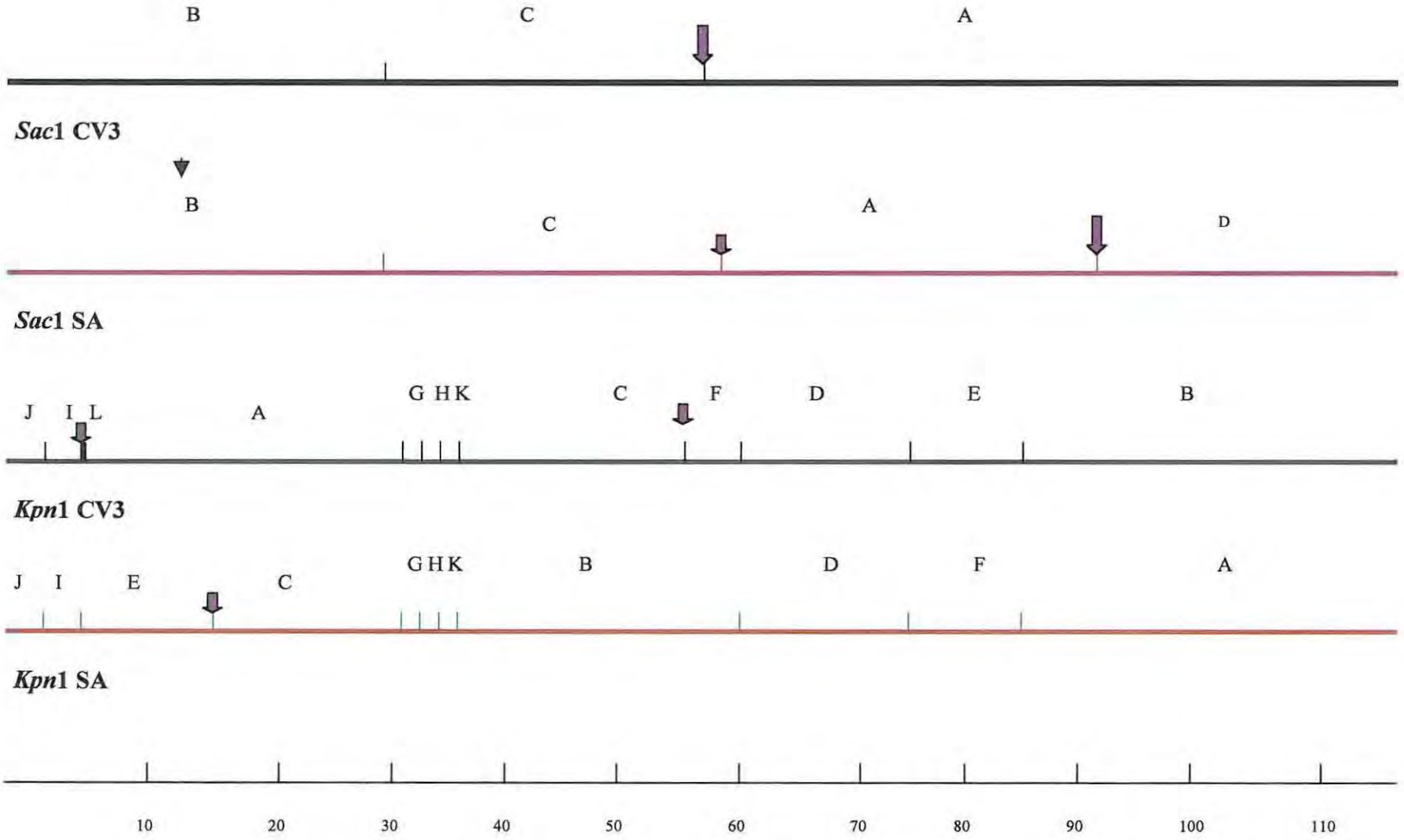
To gain more information about the position of fragments relative to each other certain specific bands were used as probes in Southern blot analysis of singly digested SA and CV3 DNA. These results helped confirm the positions of the fragments in the physical maps. All the data produced from the digests and hybridisations were incorporated to construct maps of overlapping fragments, which agreed with every hybridisation and double digest fragment size. The map for *Eco*R1 was far more difficult to construct since 24 fragments were produced. For this reason, most of the *Eco*R1 fragments were mapped using labelled probes in Southern blot analysis. Double digests with *Eco*R1 and another restriction enzyme were not attempted since too many fragments would have been produced.

The physical maps for the restriction enzymes *Bam*H1, *Kpn*1, *Sac*1, *Eco*R1 and *Xho*1 for the South African and the Cape Verde isolates of ClGV, were aligned (Fig. 30). This alignment of maps further highlighted the differences between the two isolates.

Figure 30: PHYSICAL MAPS OF THE ClGV-SA & ClGV-CV3 GENOMES FOR *Xho*1 & *Bam*H1. Differences are indicated by arrows



PHYSICAL MAPS OF THE ClGV-SA & ClGV-CV3 GENOMES FOR *Sac*1 & *Kpn*1



Although the ClGV DNA is circular, the map was linearised for clarity. The start of the map was designated as the smallest fragment containing the granulin gene. The physical maps produced in Fig. 28 thus highlight the positions of all the RE fragments for the five restriction enzymes.

CHAPTER FIVE: THE IDENTIFICATION AND CHARACTERISATION OF THE GRANULIN AND *egt* GENES

5.1) INTRODUCTION

The widespread commercial use of baculoviruses is limited because these viruses take 4 to 14 days to kill their insect hosts. During this time, serious damage to crops occurs. Genetic engineering of baculoviruses can result in an improvement in the viruses as pesticides. A variety of strategies ranging from the deletion of genes (which normally function to extend the life span of the infected insect) to the insertion of foreign genes (whose expression interferes with critical aspects of the hosts physiology), will result in the virus being able to kill the target host insect faster, cause an arrest in feeding or result in death of the insect (Chen *et al*, 2000).

In order to manipulate granuloviruses, essential genes need to be isolated and identified within the genome. Two genes of importance are granulin and *egt*. The granulin gene is highly conserved within granuloviruses and is closely related to polyhedrin of the nucleopolyhedroviruses. It is the major component of the protein capsule occluding the virion. Granulin is produced in large amounts late in the infectious cycle and it stabilises the virion allowing it to remain infectious in the environment for many years (Akiyoshi *et al*, 1985). The gene locus has received considerable attention because it can be replaced by foreign genes, which are then expressed at high levels in infected larvae (Jehle & Backhaus, 1994). The gene possesses a strong promoter, which can be linked to a foreign gene to ensure abundant expression (Eldridge *et al*, 1992).

The *egt* gene blocks the ability of the insect to moult and is a unique example of the evolution of an insect virus to allow control of the infected host at the organismal level (O'Reilly & Miller, 1990). The *egt* genes encode ecdysteroid UDP-glucotransferase enzyme that belongs to the UDP-glucosyltransferase super family. These transferases conjugate small lipophilic compounds with various sugars. *egt* is secreted into the insects haemolymph and it catalyses the conjugation of ecdysteroids, the insects moulting hormone, with the sugar moiety donated from the UDP- glucose

or UDP-galactose. This inactivates the moulting hormone and thereby prevents moulting and pupation. Larvae die in the instar in which they were infected (Wormleaton & Winstanely, 2001). Insects infected with recombinant viruses lacking the *egt* gene feed less and die more rapidly (Barrett *et al*, 1995). The alteration of *egt* expression by deletion or inactivation may provide a more effective insecticide for biological control, where the virus causes earlier mortality, reduced feeding and a decreased amount of progeny virus is produced (Riegel *et al*, 1994).

In Chapter 5 the granulin and *egt* genes are identified in the ClGV-SA isolate. This involved isolating both genes, identifying their positions in the genome, sequencing them and comparing the genes with other granulovirus granulin and *egt* genes.

5.2) MATERIALS AND METHODS

5.2.1) Granulin

5.2.1.1) The isolation of the granulin gene from the ClGV-SA genome

The granulin gene was amplified using polymerase chain reaction (PCR). Primers were designed by B.Spillings (2000) and were used to amplify a product of approximately 747 bp. Refer to Appendix 4 for the PCR reaction and thermocycler settings.

5.2.1.2) The identification of the granulin gene in the ClGV-SA genome

The position of the granulin gene was determined by probing a Southern blot of restricted ClGV DNA with a labelled granulin DNA probe. Granulin DNA was purified from the PCR reaction using the QIAquick PCR purification kit (Qiagen) (Appendix 4). Once purified, the concentration of DNA was determined using a Genequant (Pharmacia Biotech), as in Chapter 2. The DNA was then labelled with non-radioactive DIG (Roche). The hybridisation and detection of granulin was performed using the DIG protocol, discussed in Chapter 4. The gene was detected by autoradiography and its position in the ClGV genome identified.

5.2.1.3) Sequencing of the granulin gene

The sequencing of the granulin gene was performed at Horticulture Research International (HRI, UK). Sequencing reactions using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems) were set up as follows:

(1)
1.0 µl 3.2 pmol Forward primer
1.5 µl ClGV DNA (40 ng)
3.5 µl ddd water
10 µl

(2)
1.0 µl 3.2 pmol Reverse primer
1.5 µl ClGV DNA (40 ng)
3.5 µl ddd water
10 µl

The sequencing reactions were carried out using the GeneAmp PCR systems 9600 and analysed on an ABI 337 automated DNA sequencer (Applied Biosystems).

5.2.2) *egt*

5.2.2.1) The isolation of the *egt* gene from the ClGV-SA genome

The *egt* gene was amplified from the genome using PCR. Primers were designed using the *egt* sequences from two granuloviruses, *Lacanobia oleracea* GV and *Choristoneura fumiferana* GV. The sequences were aligned using the BCM search launcher sequence alignment program and two conserved regions were chosen for the forward and reverse primers. The forward primer 1 sequence was 5' GAT TGA ACA CCT CCC TG 3' and the reverse primer 1 sequence was 5' ACA CCT ATG CCG CGT G 3'. A PCR product of 1.1 kbp was amplified using PCR (the program and reaction used are presented in Appendix 4).

5.2.2.2) The identification of the *egt* gene in the ClGV-SA genome

An attempt to locate the position of *egt* in the ClGV-SA genome was made by labelling the *egt* PCR product with DIG and probing its position in the Southern blots of restricted ClGV DNA. However this technique proved to be unsuccessful. The

gene was then labelled with radioactive ^{32}P and used to probe the Southern blots, to investigate the position of the gene. Labelling was done using oligonucleotide primed synthesis. The following mixture was prepared on ice:

2.5 µl 0.5 mM dNTPs (except dCTP)
2.5 µl Klenow buffer (10 x)
1.0 µl Klenow enzyme (1 U)
4.0 µl 3000 Ci/mmol [α^{32}P-dCTP] 50 µCi

egt DNA (0.1 µg) was combined with random hexanucleotides (1 µg) to a final volume of 14 µl. The DNA was boiled for 3 minutes and then placed on ice. Once cool, the DNA was added to the mix on ice and immediately incubated at room temperature for 4 hours. The labelling reaction was stopped with 1 µl of 0.5 M EDTA. The unincorporated radioactive nucleotides were separated from the probe using the Quick spin columns (Roche) (Appendix 5). The amount of radioactivity incorporated into the probe was measured as in 6.2.3.2. The hybridisation and detection of the probe was done as in 6.2.4.

5.2.2.3) Sequencing of the *egt* gene

The pGEM-T Easy cloning kit (Promega) was used to clone the *egt* PCR products. *Eco*RV, adding 3` terminal thymidine to both ends of the vector, cuts the pGEM-T Easy cloning vector. These single 3`-T overhangs at the insertion site increases the efficiency of ligation of PCR product into the plasmid by preventing recirculisation of the plasmid and providing compatible overhangs for PCR products (Promega manual, 1998). The gene had to be cloned in order for it to be sequenced. *egt* PCR products were ligated into the pGEM- T Easy plasmid vector using the reaction below:

	STANDARD REACTION	POSITIVE CONTROL	BACK-GROUND
2 x rapid ligation buffer	5 µl	5 µl	5 µl
pGEM – T EASY VECTOR (50 ng)	1 µl	1 µl	1 µl
PCR product (insert ratio 3:1)	x	-	-
Control insert DNA	-	2 µl	-
T4 DNA Ligase (3 Weiss units/µl)	1 µl	1 µl	1µl
ddd water to a final volume of	10 µl	10 µl	10 µl

The reaction was mixed and incubated overnight at 4°C. The ligation was then transformed into *E.coli* DH5α cells using heat shock (Appendix 2). Insertional inactivation of the α-peptide coding region of the β-galactosidase gene allows recombinant clones to be directly identified by colour screening. Transformants were screened for blue/white colonies on LB/AMP media containing X-gal and IPTG. White colonies were picked and subcultured in 5 ml LB/Amp media overnight at 37°C. Plasmid DNA was extracted using alkaline lysis (Appendix 2).

The plasmid vector contains duplicate restriction sites flanking the multiple cloning region and these sites allow for the release of the insert by digestion with a single restriction enzyme (see Appendix 4). Recognition sites for *Eco*R1 flank the multiple cloning sites and this enzyme was used for digestion of the plasmid. 10 μl of plasmid was digested with 2 μl of *Eco*R1 (20 units), 2 μl of Buffer H (10 x) and 6 μl of double distilled water at 37°C for 2 hours. The digest was then electrophoresed on a 1 % agarose gel to ensure that the plasmid and insert were both present. Once the gene was cloned, it was prepared for sequencing by subculturing the clone overnight in 5 ml LB/AMP, at 37°C. The plasmid DNA was purified using the QIAprep spin columns (Qiagen). The DNA was then amplified by thermocycling and further purified using the Zymo columns (Zymogen). These methods are discussed in Appendix 4. Sequencing was done by Mrs Anna Clark using the ABI 3100 Genetic Analyser at Rhodes University.

The Genetic Analyser generates approximately 500 bp of sequence during a sequencing reaction. Since *egt* was 1 376 bp in size, the gene had to be sequenced in stages. Another forward primer was then designed to cover the region from the middle of the gene to the reverse primer. The Primer 3 program was used for the design of the *egt* Forward primer 2 (5` GAT ACC TGT GT CGC CTT TC 3`). Using the Forward primer 2 and the Reverse primer 1, a smaller PCR product (685 bp) of the *egt* gene was obtained, which was also cloned into pGEM plasmid vector and sequenced as above. A region of 200 bp in the middle of the *egt* gene remained unsequenced and another set of primers, to include this region, was designed using the Primer 3 program. These primers were designated Forward primer 3 (5` GGT ATC AGC TCA CTC AAA GG 3`) and Reverse primer 3 (5` GGT TGG ACT CAA GAC GAT AG

3`). Using these primers the unsequenced region was isolated by PCR, cloned into pGEM and sequenced. 156 bp before the start of the first forward primer remained unsequenced. A forward primer was designed, to amplify the region 66 bp before the start codon (ATG) of the gene, using the LoGV *egt* sequence. This region was amplified by PCR using the Forward primer 4 (5` GAT GAC CGC TTC GAG CCA TGA 3`) and Reverse primer 3; and it was cloned and sequenced. The various sequences were combined to produce a preliminary ClGV-SA *egt* sequence. Refer to Fig. 31 for the primers designed for the isolation of egt.

Figure 31: Forward and reverse primers designed for *egt* amplification.

5.3) RESULTS

5.3.1) Granulin

5.3.1.1) Isolation of the granulin gene

The granulin gene was successfully amplified from the ClGV-SA genome using PCR. A product of 747 bp was isolated (Fig. 32).

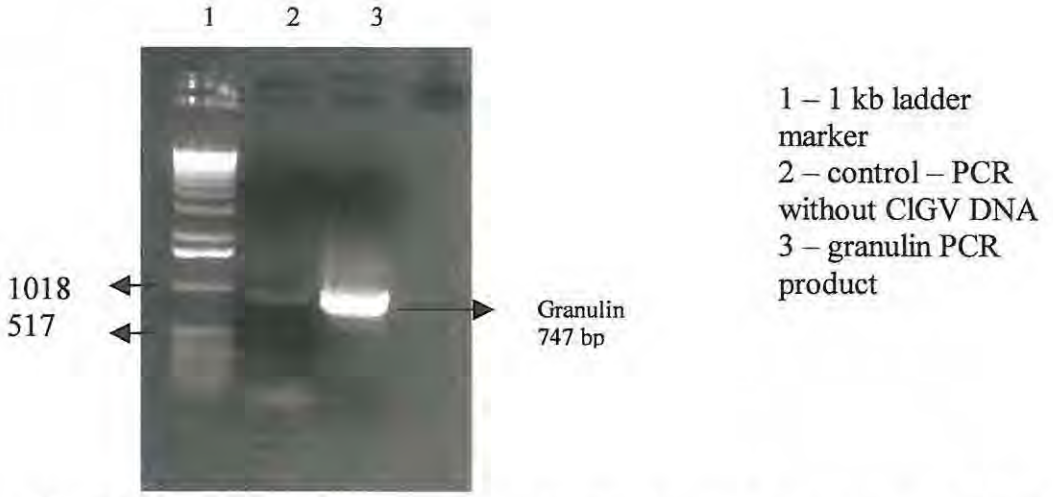
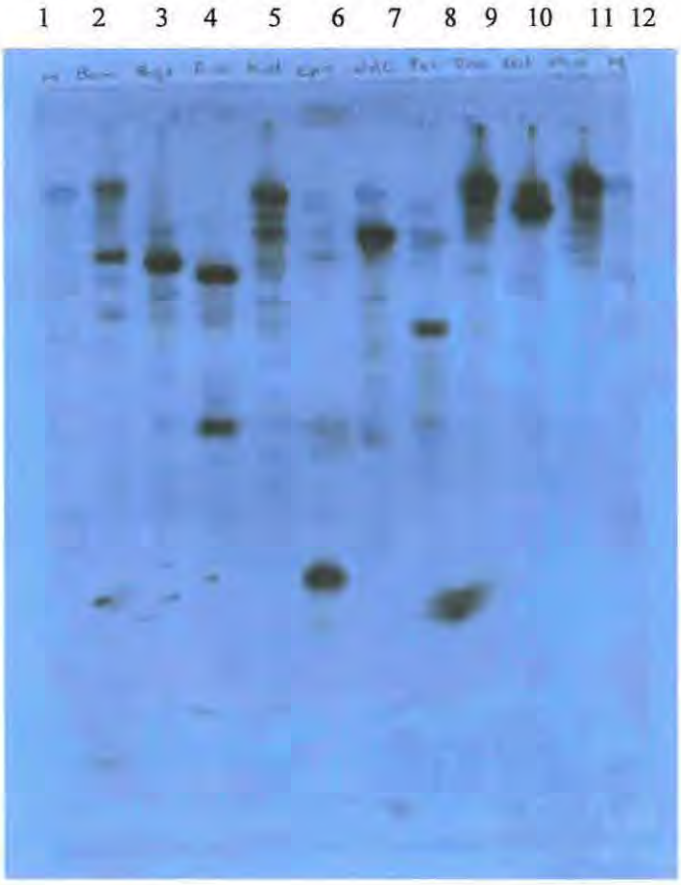


Figure 32: PCR product of the granulin gene (747 bp) on a 1 % agarose gel. A 1 kb ladder was used as a molecular weight standard (Appendix 4). An overflow of DNA occurred from lane 3 into lane 2.

5.3.1.2) The identification of the granulin gene in the ClGV-SA genome

In each restriction enzyme digest of ClGV DNA, the granulin probe hybridised to a particular fragment or fragments producing a signal on the X-ray film (Fig. 33). These fragments thus contained portions of the granulin gene, e.g. the probe hybridised to fragment A and N in the *Eco*R1 digest, fragment J in the *Kpn*1 digest, fragments C and A in the *Bam*H1 digest and in the *Xho*1 digest, it bound to fragment A. These fragments thus contained the granulin gene sequences.



1 – 1 kb ladder marker
2 – *Bam*H1 digest
3 – *Bgl*II digest
4 – *Eco*R1 digest
5 – *Hind* III digest
6 – *Kpn*1 digest
7 – *Nde*1 digest
8 – *Pst*1 digest
9 – *Sac*1 digest
10 – *Sal*1 digest
11 – *Xho*1 digest
12 – 1 kb ladder marker

Figure 33: The position of the granulin gene in the ClGV-SA genome. The DIG-labelled granulin probe hybridised to a particular fragment or fragments in each RE digest. A photograph of the corresponding RE digests of ClGV-SA DNA is visible beneath the X-ray.

5.3.1.3) The sequencing of the granulin gene

The sequence of the granulin gene is presented in Fig. 34. The ClGV-SA granulin sequence was aligned with the ClGV-CV3 granulin sequence. There exist only six nucleotides that differ between the two sequences (highlighted in red). Fig. 35 presents the granulin amino acid sequences for both ClGV-SA and ClGV-CV3. No differences between the amino acid sequences were noted.

Figure 34: Granulin nucleotide sequence (Top ClGV-CV3, bottom ClGV-SA)

```
1   ATGGGATATAACAAATCTTTGAGGTACAGCCGTCACGACGGTACCACTTG
1   ATGGGATATAACAAATCTTTGAGGTACAGCCGTCACGACGGTACCACTTG

51  TGTAATTGACAACCACCATTTGAAGAGCTTGGGAGCCGTGTTACACGATG
51  TGTAATTGACAACCACCATTTGAAGAGCTTGGGAGCCGTGTTACACGATG

101 TCAGACGTAAAAAAGATCGCATCCGTGAAGCGGAATACGAGCCCATTATC
101 TCAGACGTAAAAAAGATCGCATCCGTGAAGCGGAATACGAGCCCATTATC

151 GATATCGCCGATCAATACATGGTGACCGAGGATCCATTTCGTGGACCCGG
151 GATATCGCCGATCAATATATGGTGACCGAGGATCCATTTCGTGGACCCGG

201 CAAGAATGTAAGGATCACCCTTTTCAAGGAAATTAGACGTGTCCACCCAG
201 CAAGAATGTAAGGATCACCCTTTTCAAGGAAATCAGACGTGTCCACCCAG

251 ACACAATGAAGCTGGTATGCAACTGGAGCGGCAAAGAATTCCTTCGCGAG
251 ACACAATGAAGCTGGTATGCAACTGGAGCGGTAAAGAATTCCTTCGCGAG

301 ACTTGGACCCGTTTCATCTCTGAAGAATTTCCCATCACCACAGACCAAGA
301 ACTTGGACCCGTTTCATCTCTGAAGAATTTCCCATCACCACAGACCAAGA

351 AATTATAGATTTGTGGTTTGAGCTTCAGCTACGACCAATGCACCCTAACC
351 AATTATAGATTTGTGGTTTGAGCTTCAGCTACGACCGATGCACCCTAACC

401 GTTGTTACAAATTCACTATGCAGTATGCTCTCTGTGCCCATCCCGATTAT
401 GTTGTTACAAATTCACTATGCAGTATGCGCTCTGTGCCCATCCCGATTAT

451 GTCGCTCACGATGTGATCCGCCAGCAGGATCCCTAGTATGTAGGACCTAA
451 GTCGCTCACGATGTGATCCGCCAGCAGGATCCCTATTATGTAGGACCTAA

501 CAATATCGAGCGTATCAATCTTTCCAAGAAGGGTTTCGCTTTCCCACTCA
501 CAATATCGAGCGTATCAATCTTTCCAAGAAGGGTTTCGCTTTCCCACTCA

551 CATGCCTACAGTCCGTCTACAATGACAACTTTGAGAATTTCTTTGATGAC
551 CATGCCTACAGTCCGTCTACAATGACAACTTTGAGAATTTCTTTGATGAC

601 GTTCTGTGGCCGTATTTCCACCGTCCCCTGGTGTATGTAGGTACTACGTC
601 GTTCTGTGGCCGTATTTCCACCGTCCCCTGGTGTATGTAGGTACTACGTC

651 TGCCGAAATTGAGGAGATCATGATTGAAGTTTCTCTGTTGTTCAAGATCA
651 TGCCGAAATTGAGGAGATCATGATTGAAGTTTCTCTGTTGTTCAAGATCA

701 AGGAGTTTGCACCCGACGTGCCCCTATTCACCGGTCCAGCCTATTAA
701 AGGAGTTTGCACCCGACGTGCCCCTATTCACCGGTCCAGCCTATTAA
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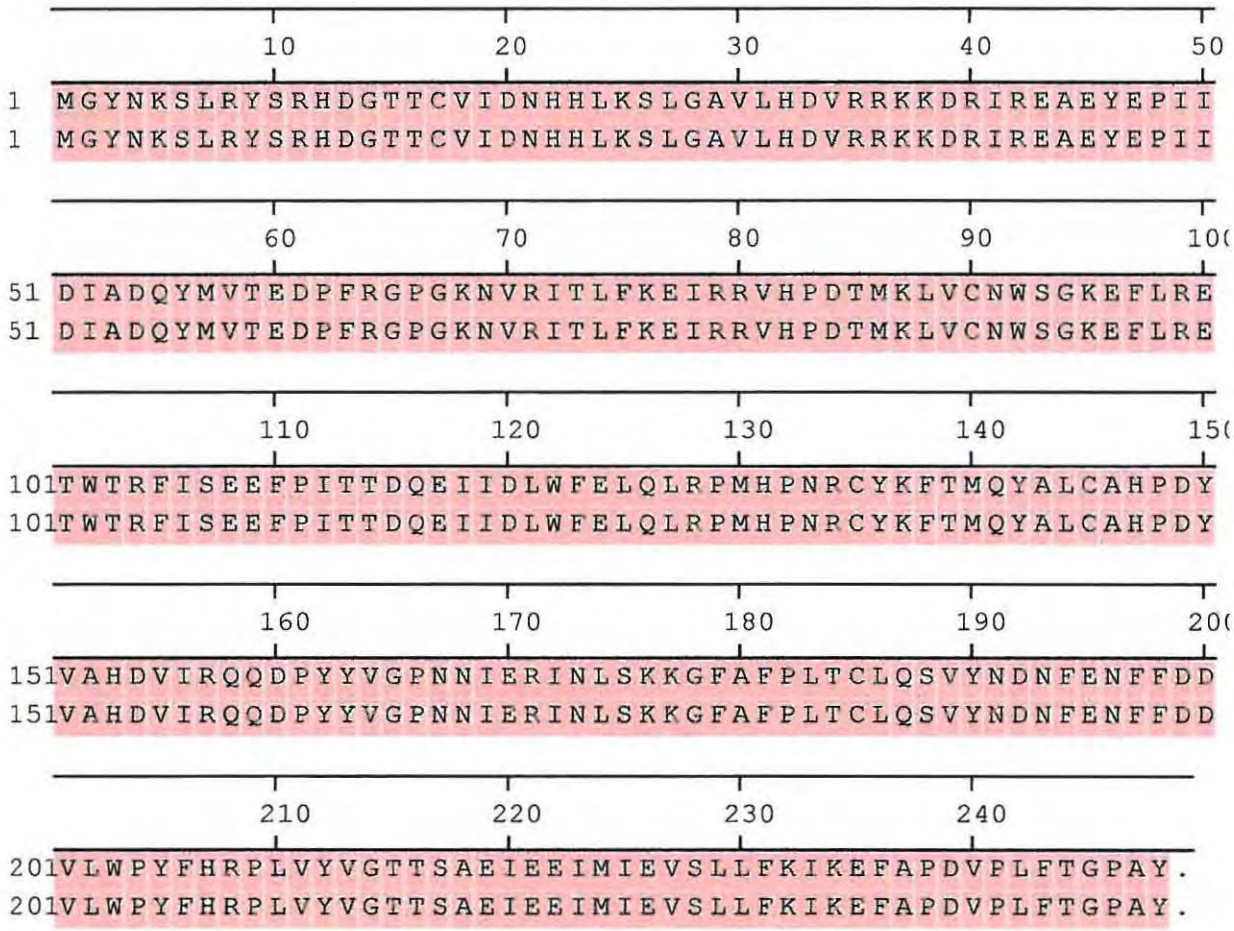
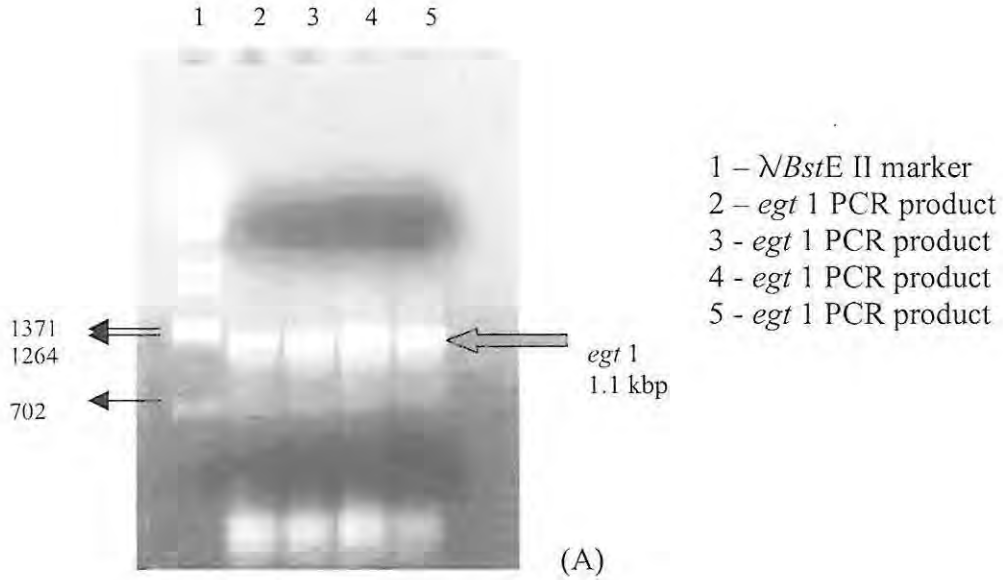
Figure 35: Granulin amino acid sequence (top ClGV-CV3, bottom ClGV-SA)

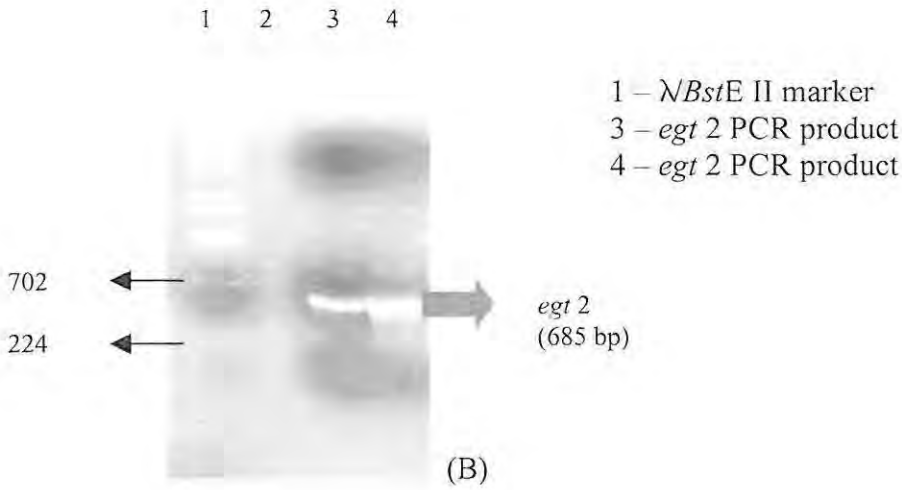


5.3.2) *egt*

5.3.2.1) The isolation of the *egt* gene

The *egt* gene was successfully amplified from the genome using PCR. Fig. 36 (A) shows the *egt* PCR product of 1.1 kbp in size. Three smaller pieces of the gene were also amplified by PCR to enable to the gene to be sequenced. Fig. 36 (B) and (C) presents the 685 bp and 490 bp fragments of the gene.





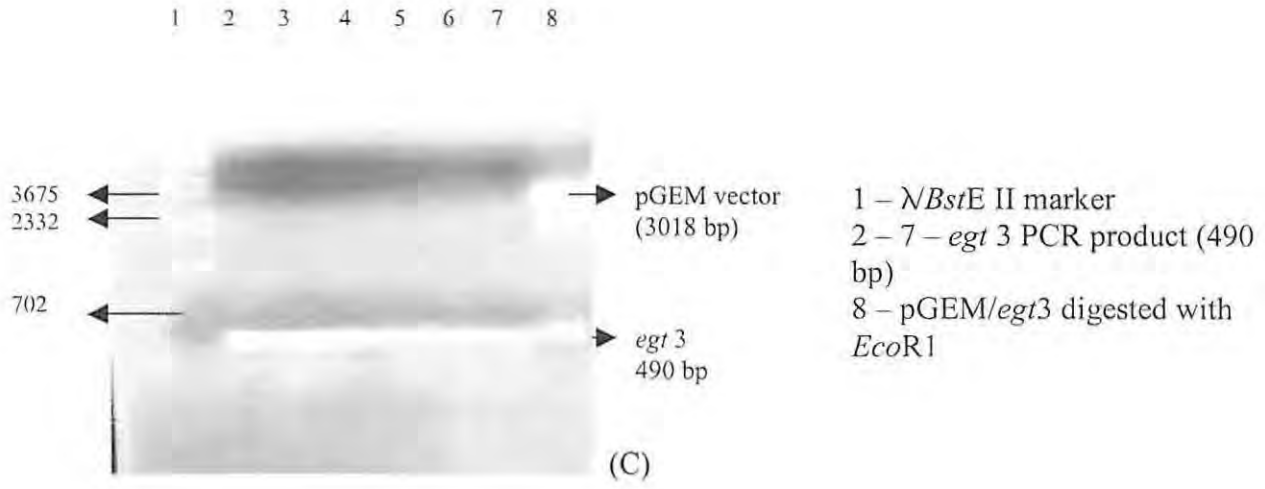


Figure 36: *egt* PCR products on 1 % agarose gels. (A) 1.1 kbp PCR product (B) *egt* 2 (685 bp) PCR product (C) *egt* 3 (490 bp) PCR product and pGEM/*egt*3 clone

5.3.2.2) The sequence analysis of *egt*

The PCR products of the *egt* gene were each cloned into pGEM-T Easy vector. Fig. 37 shows the pGEM/*egt*2 clone. Each fragment was then sequenced. The sequence information generated was combined to produce a sequence for *egt* (Fig. 38). The sequence was then translated, but there appeared to be many stop codons and possible errors in the sequence. Therefore the sequence produced is a preliminary sequence of the ClGV-SA *egt* gene. The gene needs to be sequenced in the opposite direction, with suitable overlap, to produce a complete sequence of the gene.

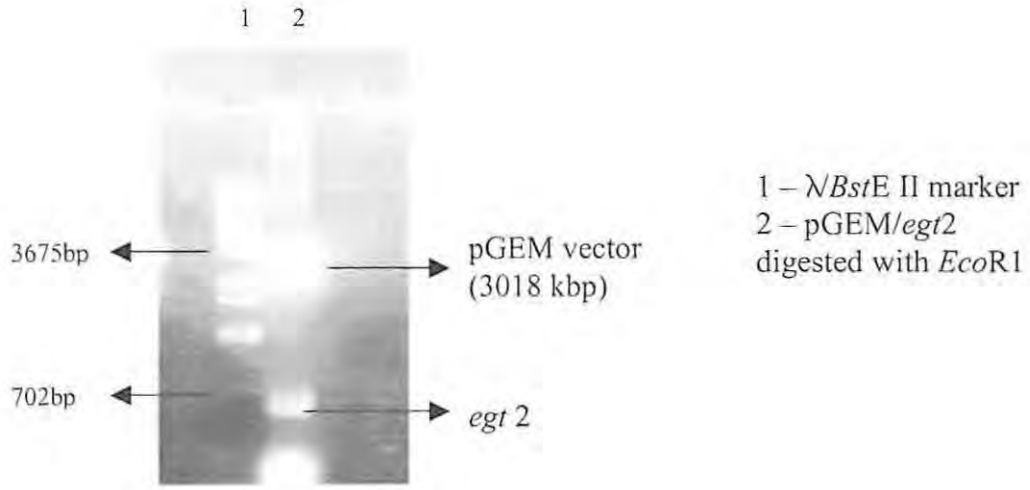
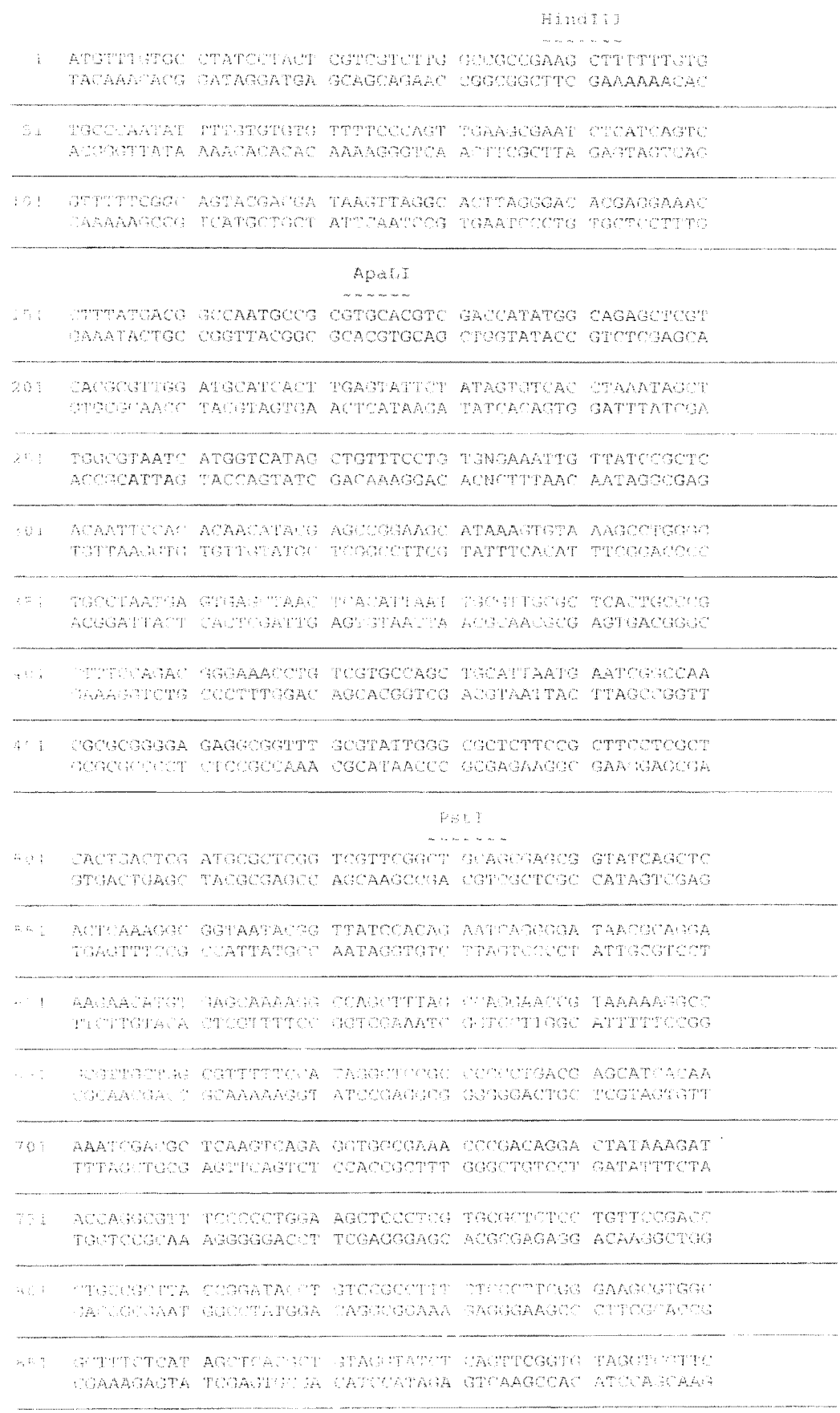
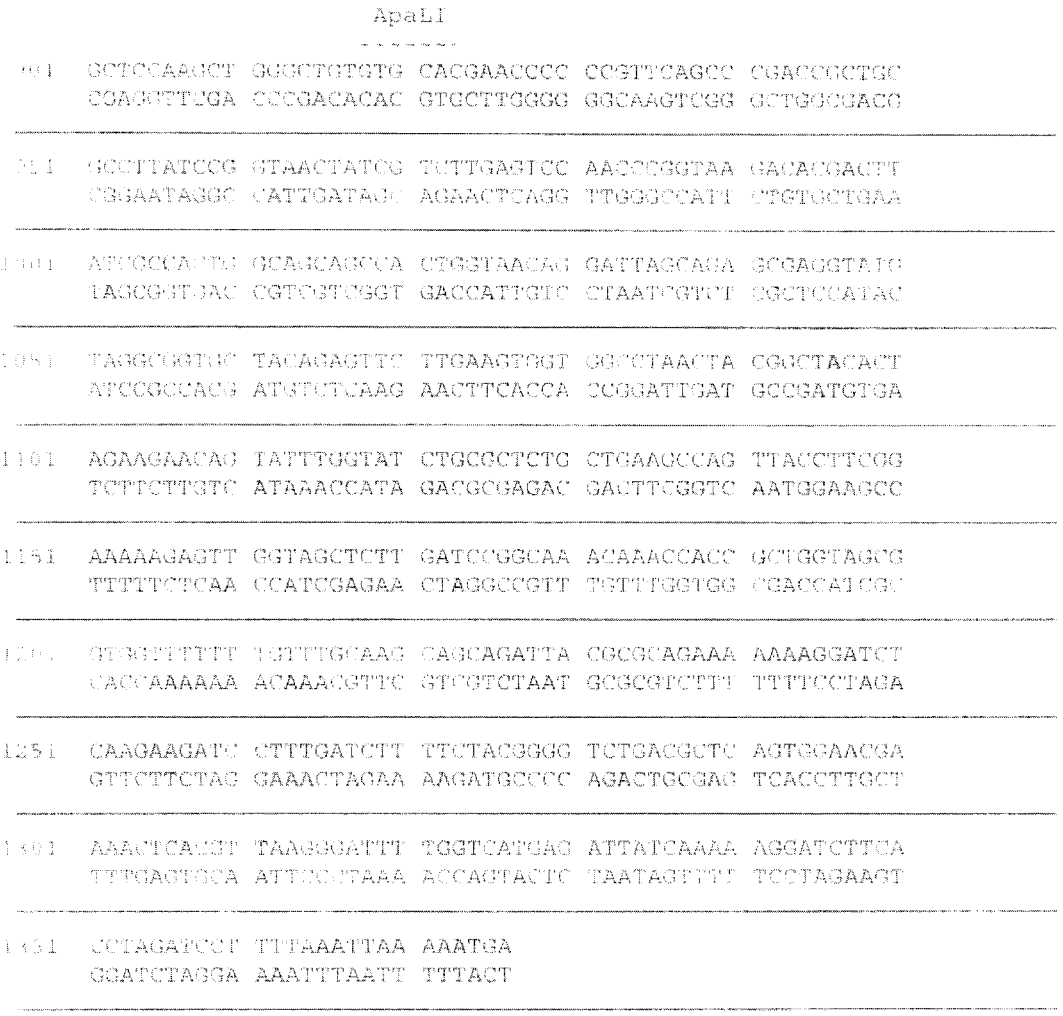


Figure 37: The *egt*2 PCR product cloned into pGEM-T Easy plasmid vector.

Figure 38: The *egt* sequence of ClGV-SA





To confirm that the gene isolated was *egt*, the sequence generated was aligned with other granulovirus *egt* sequences, namely *Adoxophyes honmani* granulovirus (AhGV), *Adoxophyes orana* granulovirus (AoGV), *Epinotia aporema* granulovirus (EaGV), *Cydia pomenella* granulovirus (CpGV), *Choristoneura fumiferana* granulovirus (CfGV) and *Lacanobia oleracea* granulovirus (LoGV) (Fig. 39). There was alignment of the ClGV-SA *egt* sequence with the other granulovirus *egt* sequences, suggesting that the gene isolated was in fact *egt*. A consensus between the sequences is indicated in Fig. 39. However there are regions of non-alignment suggesting that the ClGV-SA sequence is incomplete or has errors; and requires further sequencing.

Figure 39: Multiple sequence alignment of the *egt* genes from 7 granuloviruses using ClustalW multiple sequence alignment & Boxshade.

```
Page 1.1
          1              15 16             30 31             45 46             60 61
    1 AhGV  --------------- --------------- --------------- ATGTAT---TCGTTG ATTGTGTTGT
    2 AoGV  --------------- --------------- --------------- ATGTAT---TCGTTG ATTGTGTTGT
    3 CfGV  --------------- --------------- --------------- ATG--T---TCGTGC AACTAATACT
    4 logv  --------------- --------------- --------------- ATGTTTATATCTATC CTACTTTTAC

    5 CpGV  ATGGGACGATACACT CCAAATGGGGCACAA CCCAATTCGCTCAAC ATGTTT--CGCAATA ATTTGGTCCT
    6 AFgv  --------------- --------------- --------------- ATGTGGACAGCGATC GTA--GTTGT
    7 CFNPV --------------- --------------- --------------- ATGGCTTCTTTACTT ATTGCATTGA
    8 MbNPV ---------ATGGGT CACCTACATATTGTC CATTGGCGTCTCACC ATGAACGGAGCTATT GCTGCATTGT

    9 ClGV  --------------- --------------- --------------- ATGTTTGTGCCTATC CTA--CTCGT


Page 2.1
          91             105 106            120 121            135 136            150 151
    1 AhGV  -----TGTCTCGTCC GCTAGAATATTATGC GTTTTT-CCAA-TAC CGTCATATAGTCATC ATTTGGTTTA
    2 AoGV  -----TGTCTCGTCC GCTAGAATATTATGC GTTTTT-CCAA-TAC CGTCATATAGTCATC ATTTGGTTTA
    3 CfGV  T--TTTGTTTGTTCG TCAAATATTTTGTGT GTGTTT-CCCA-CAC CGGCGTTGAGTCATC AGTCTGTGTT
    4 logv  ------ATTTTGTGT GCCAACATTTTGTGT GTTTTC-CCCA-CTC CTGCGTACAGTCACC AATCTGTTTT

    5 CpGV  CACTTTGTACACACG GCCAATATTTTGTGT GTTTTC-CCCA-CGC CCGCGTTCAGTCACC AATCCGTGTT
    6 AFgv  --------CGCCGCG CACAATATCCTATGC GTGTTT-CCCA-CAC CCGCATATAGTCACA ACTCGGTTTT
    7 CFNPV C-----GCGCAAACC GCGAATATTCTAGCG GTGCTG-CCCA-CGC CAGCTTATAGTCACC ACGCTGTGTA
    8 MbNPV CATCAGCAACATGCA GTCAGGATCTTGGCG GTGTTT-CCGA-CTC CGGCGTACAGCCATC ACAGCGTGTT

    9 ClGV  -----TTTTTGTGTG CCCAATATTTTGTGT GTGTTTTCCCAGTTG AAGCGAATC-TCATC AGTC-GTTTT


Page 3.1
          181            195 196            210 211            225 226            240 241
    1 AhGV  TGGTTAAAAACGGAC ATAATGTTACCGTCA TAA----CTCC---- ---------CATGCC TAGACATGTC
    2 AoGV  TGGTTAAAAACGGAC ATAATGTTACCGTCA TAA----CTCC---- ---------CATGCC TAGACATGTC
    3 CfGV  TAGTTATTGCTGGAC ACAACGTTACCGTGA TCA----CACC---- ---------TATGCC GCGTGGAGTT
    4 logv  TATCTTGGGCCGGAC ACAACGTGACGGTGA TTA----CACC---- ---------TATGCC GCGTGCCGTC

    5 CpGV  TGGCCAAGGCTGGCC ACAACGTTACCGTGA TTA----CCCC---- ---------ATTGCC GCGCCGTGTC
    6 AFgv  TGGTAAAGGACGGTC ACAATGTCACAGTTA TTA----CCAC---- ---------AATGCC CAGAAACATT
    7 CFNPV TTGCAAAAAATTGCC ACAATGTAACCGCTG TGA----AGCCGCGG CTACTGGATTACGCC TTGCTAAATC
    8 MbNPV TGGCCGAACGCGGCC ACGACGTAGTGGTCA TTAAAT-CTACCGAC AGAATCAACTATGCC AACAGAAATC

    9 ClGV  TAGGCACTTTGGGAC ACGAGGAAACTTTTT TGATGGCCAACCC-- ---CGCCTGCAGGTC GA-CCATATC


Page 4.1
          271            285 286            300 301            315 316            330 331
    1 AhGV  GAAATCGACAC-ATC A--AAGAACACCTT- -------GAAC---G TGTACAG--CG-CTT TAATGAGCAC
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http://dot.imgen.bcm.tmc.edu:9331/cgi-bin/multi-align/multi-align.pl 02/04/2002

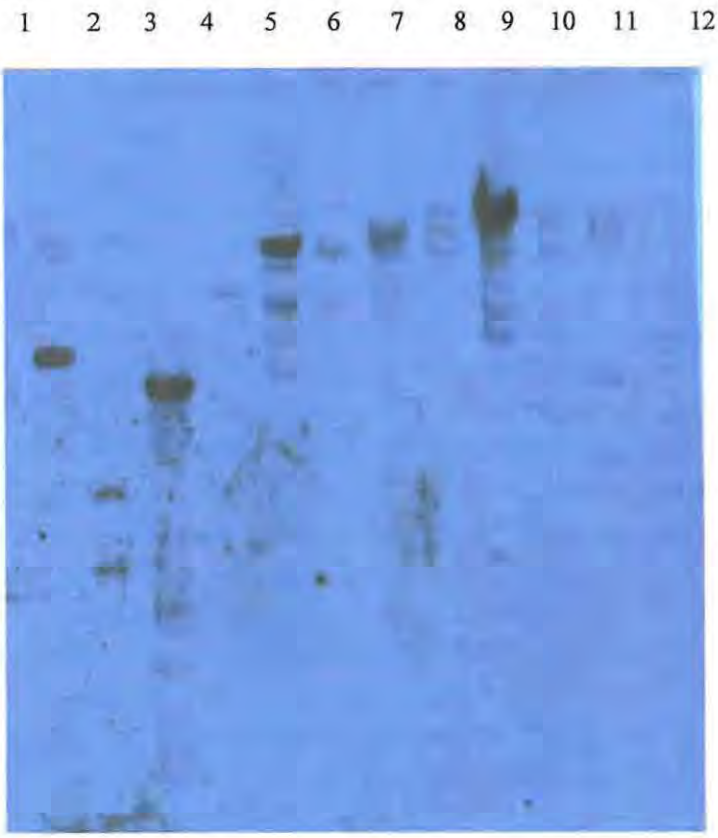
```
   2 AoGV  GAAATCGACAC-ATC A--AAGAACACCTT- -------GAAC---G TGTACAG--CG-CTT TAATGAGCAC
   3 CfGV  GAAATAGATTG-TTC A--ACTAAAAACGTA TT-CGT-GAATTTGG TGAAAAATTCGACAT CATTGAAAAA
   4 logv  CAAGTTGTTAG-CTC GCTATCTGTGCATTA TT-TTAACAATCTAA TCAAGAATTCTACAA TGATCAAGAA

   5 CpGV  GAAATTGACTGCAGC AAATCGTTGACTGTG TT-CAATGAGT-TAG TGAAGAAGTCGTCGT TGTTCAAAGT
   6 AFgv  CAAGTTGACTG-CTC GTTGCCAGGACACTA TTTCCAGGAAT-TGG TCAAAAACTCGACGA TGATCAAGAA
   7 CFNPV CAAATTGACGCCGAC ATGTCGTTGGAGCAA TACCA--GAAGCTAA TGGCCGGTTCAGGCG CGTTCCGCAA
   8 MbNPV GAAATTGACGC-TTC ACTCTCTCAAGAATA CTATG-GCCGTCTAA TGCGTCACGCCGGAG TTTTTCGGAA

   9 ClGV  C--GTTGGATGCATC AC-TTGAGTATTCTA TA-----GTGTCACC TAAATAGCTTG-GCG TAATCATGGT


Page 5.1
           361         375 376         390 391         405 406         420 421
   1 AhGV  AAATTTTTGT---TG CAGAACAATACCGTG ATTTGTTAAATATGG TCGTCGATCAATTTA ACAGCGATGC
   2 AoGV  AAATTTTTGT---TG CAGAACAATACCGTG ATTTGTTAAATATGG TCGTCGATCAATTTA ACAGCGATGC
   3 CfGV  AATTGACAGTAACTG CTGAAAATTATACAC CAATAATAGATATGG TGGTGGAGCAGATAA AAAGTTACAA
   4 logv  AAACCACCGTGACAA AGGAAAATTATATGG GACTGATTAATTTGG TGGCGCATGAAATAA AAAGTCCTAA

   5 CpGV  AGAGCACAGTGACCG CCGACAATTACACAC CGCTGGTGGACATGG TGGTAGCCCAGTTCC AAAATAAAAA
   6 AFgv  AAACTACGGTAACCA AGGAAAATTACATGG GTTTGGTTGATATGG TGGTGCGGGAAATGC AACACGAAAA
   7 CFNPV AAACCACCGTCACTG CGGACAATTACATGA GCCTGATAGAAATGT TTAAGGACCAATTTG ACAACGCCAA
   8 MbNPV GTTCCACGGTCACCG CCCACAACTACATGG GACTGGTGCGCATGA TGAGCGATCAATTTG ATTTGCCCAT

   9 ClGV  AAATTGTTAT--CCG CTCACAATTCCACAC AAC--ATACGAGCCG -GAAGCATAAAGTGT AAAGC-CTGC


Page 6.1
           451         465 466         480 481         495 496         510 511
   1 AhGV  CAACACA-AATGTCA AATTTGATTTGATAG TATGCGAGTCCATGC TGAACATTAACCTAT ACTTTGGACA
   2 AoGV  CAACACA-AATGTCA AATTTGATTTGATAG TATGCGAGTCCATGC TGAACATTAACCTAT ACTTTGGACA
   3 CfGV  AAACAAA-GATAATA ATTTTGATTTGGTTG TATGTGAAGCATACC TTGATATAATTTTAA TATTTGGTCA
   4 logv  AAACAAG-GGTAACA AGTTCGACTTAATTG TTTGTGAAGCTTATG TCAGTTATATCTTGG TGTTTGGAGC

   5 CpGV  AAACAAG-GCCAACA TGTTTGATTTGGTGG TGTGCGAAGCGTACC TTAGTCTCAACCTAA TTTTTGGTCA
   6 AFgv  AAA---G-GACCAGA TGTTTGACTTGGTAG TGTGTGAGGCGTATA TTAGTTACATACTGG TGTTTGGGTA
   7 CFNPV CTCCA----ACCGGA CCTTTGACGCCGTGG TGGTGGAAGCGTCCG CCGACTACGAGCTTG TGTTTGGGCA
   8 MbNPV AAGCACACAAGCACA AATTTGATTTGTTAA TAACCGAGGCGTACA TCGATTATCCTCTTG TTTTCTCACA

   9 ClGV  TAACTCACATTAATT GCGTTGCGCTCACTG CCCGCTTTCCAGACG GGAAACCTGTCGTGC CAGCTGCATT


Page 7.1
           541         555 556         570 571         585 586         600 601
   1 AhGV  TA---ATTTGGATCT CTTC--GGGA----C GAGCCACCAACGACA ATTATAACACCCTAT TAAACAAAAC
   2 AoGV  TA---ATTTGGATCT CTTC--GGGA----C GAGCCACCAACGACA ATTATAACACCCTAT TAAACAAAAC
   3 CfGV  TA---ATAAAATTTT CATC--CGGC----T ATGGTACCAACGAAA ATTTTAAAAC---AA TGAACGCGGA
   4 logv  TG---ATCCAGTTCT CGTC--GGGC----T ACGCCATACCTGAAA ACTTTGAAAC---TG TGGGCGGCGA

   5 CpGV  TC---ATTCGTATCT CTAG--TGGG----C ACGGCACTACCGAAA ATTTTGACACC---A TGGGCG---C
   6 AFgv  TG---ATACAGTTTT CGTC--TGGC----T ACGGATTGGTGGAAA ACTTTGAAAC---TA TGGGTA---T
   7 CFNPV TA---ATACAAATCG CGCC--GGGT----T ACGGTTTGGCCGAGA ACTTTGACGCC---G CCGGCGCCGT
   8 MbNPV TG---GTGCAAATTT CATC--GGGA----T ACGCTGTGGCTGAAA ACTTTGAAAC---TA TGGGAG---C

   9 ClGV  GGGAGAGGCGGTTTG CGTATTGGGCGCTCT TCCGCTTCCTCGCTC ACT--GACTCG---A TGCGCTCGGT


Page 8.1
           631         645 646         660 661         675 676         690 691
   1 AhGV  ATATATCCCAATGTA TTTAACACATTGAAT TGTGAAAATGAAGAA AAATGTCACGACG-A CGA-------
   2 AoGV  ATATATCCCAATGTA TTTAACACATTGAAT TGTGAAAATGAAGAA AAATGTCACGACG-A CGA-------
   3 CfGV  GTGTATCCCAACCTG TGGCGGTCA---AAC TTTTCAAATGAA--A ATATTGAACAAG--- CGC-------
   4 logv  AAGCACCCAAACATT TGGCGGTCT--GATT TCAGCAAGTCTA--- ACTTTGAACAACTGA TGA-------

   5 CpGV  GTGTATCCGAGTAGC TGGCGCTCC---ACA TTTTCCACTAAC--A AAACCCAAATAATGG AGA-------
   6 AFgv  ACACATCCCAACTTG TGGAGATC------- TTCGCAA-------- AATGCTAGCTAC--- -GA-------
```

```
    7 CFNPV CACTACCCCAACATT TGGCGCAGCA--GCT TCAGCGG--CGAGGC GGCGGGCGCGCTCAG CGA-------
    8 MbNPV TACTATCCTAATTTG TGGCGGGACA--AGT TTAGCGGACTAAACG TGTGGAAACAATCAA TGAAATGTAC

    9 ClGV  TATCAGCTCACTCAA AGGCGGTAATACGG- TTATCCACAGAA--- TCAGGGGATAACGCA GGA-------


Page 9.1
            721         735 736         750 751         765 766         780 781
    1 AhGV  A-GAATGGAATAAAT TTGACGTCATACAAC ---ACGATTTACTCA CAAAACAT-TTTGGT CAAGACATAC
    2 AoGV  A-GAATGGAATAAAT TTGACGTCATACAAC ---ACGATTTACTCA CAAAACAT-TTTGGT CAAGACATAC
    3 CfGV  A-GT-TGAAACAAAT A------CAGAATAT ---TCGATT-----A CAAAAATTGTTTGGA GACCGCACTT
    4 logv  ACGAATGGGCTCTAT TGGAAAAGGAACAAG ---AGAACATGTTGA -AACGCGACTTTGGA TACCAC---C

    5 CpGV  ACGAATGGAAGAAAT TGGAGAGGGTGCAGG ---A-GACACGGTTA AAACAGGTGTTTGGA GGTGGTAG-C
    6 AFgv  A-GAGTGGGCCATGC TTGAGCGGGCGCAAC ---ACGATATCATGC GGGATAAT-TACGGA ACACGTGTAC
    7 CFNPV ACGAATTTGAGCTGC TGGCGTCGCAGCGAT CCAACGAGCTGCTGA -AACAACAGTTTGGT CTTGACACGC
    8 MbNPV ATGAATTTAGTAAAT TGGCC--GGACGAAC AGAATGCACTATTGA -AACGCCAGTTTGGC GAGAGCACTC

    9 ClGV  AGGCCAGCAAAAGCC AGGAACCGTAAAAAG G--CCGCGTTGCTGG -----CGTTTTTCCA TAGGCTCCGC


Page 10.1
            811         825 826         840 841         855 856         870 871
    1 AhGV  GAAGAAGAGAGTCAA ATTGTTGTTTATCAA --TACCGCGTCCCAT TTTGATAACGACAGA -----CCCAT
    2 AoGV  GAAGAAGAGAGTCAA ATTGTTGTTTATCAA --TACCGCGTCCCAT TTTGATAACGACAGA -----CCCAT
    3 CfGV  GCAGCAAAGTGTGAA ATTGTTATTTGTCAA --TGTACCCCACGTG TTTGACAGCGACAGA -----CCTGT
    4 logv  GAAATCTAGGGTCTT AATGTTGTTTATTAA --CGTTCCTGCAGTG TTCGATAATAACAGA -----GATGT

    5 CpGV  GAAAAAACGGGTGGT GCTACTGTTGGTGAA --CGTACACCCTGTG ATGGATAATAACAGA -----CCAGT
    6 AFgv  GAGACAGCGCGTGTT AATGTTATTCATAAA --CGTGCCGGCTATT TTTGACAACAACAGG -----CCGGT
    7 CFNPV ACGTGACAACGTGCA ACTTTTACTACTCAA --CCTGCACCCGGTG TACGACAACAACCGT -----CCGGT
    8 MbNPV GCGCAATAGAGTCGA GCTGTTGTTTGTTAA --CACACACGCCATA TTCGATAACAATAGG -----CCGGT

    9 ClGV  AAAATCGACGCTCAA GTCAGAGGTGGCGAA ACCCGACAGGACTAT AAAGATACCAGGCGT TTCCCCCTGC


Page 11.1
            901         915 916         930 931         945 946         960 961
    1 AhGV  TGGGCGG---TCTTC ATTTGACAACACCGC CGCCTATATTGAGCG ATGTCGATGTGAAAC TAACCAATTT
    2 AoGV  TGGGCGG---TCTTC ATTTGACAACACCGC CGCCTATATTGAGCG ATGTCGATGTGAAAC TAACCAATTT
    3 CfGV  TGGGTGG---TATTC ATTTAAAAAAACCAC GACCGGT-----GCG ----CGATATAAAAT TAATAGAATT
    4 logv  TGGGTGG---AATTC ACCTCAAAAAACCAA GAACAGT-----GCG ----CGACTCACGTT TGCTCTCGTT

    5 CpGV  TGGGGGG---GCTGC ATTTGAAACGAGCTC AATCGATA----AAG -----GACGAGGAGT TGCGCGCCTT
    6 AFgv  TTGGCGG---TATGC ATCT---ACGATCTC CCACTAA-------G -----GATGTG-AG- -------CTT
    7 CFNPV TGGGCGGCGGGCTGC ATTTGTCGCAGGCGC CGTCGCACAAGTTAA CCGCCGCGCTCGAGC G--CCGGCTC
    8 MbNPV TGGGCGC---ATTAC ATTTG-CACGATAAG CGACCGGAT--AGCA TGTACGGTATG--GT T-CGTGAATT

    9 ClGV  CTGTTCCGACCCTGC CGCTTACCGGATACC TGTCCGCCTTTCTCC ----CTTCGGGAAGC GTGGCG-CTT


Page 12.1
            991        1005 1006        1020 1021        1035 1036        1050 1051
    1 AhGV  -ACAATAATATATGT CAGTTTCGGATCAAA CAATTTCAACAA-CA CCA-TATTGAAAAAC GTAT--TCGT
    2 AoGV  -ACAATAATATATGT CAGTTTCGGATCAAA CAATTTCAACAA-CA CCA-TATTGAAAAAC GTAT--TCGT
    3 CfGV  -AATATAATTTATGT TAGCTTTGGATCTAT ---TTTGGACGC-CG CTGCTATGGACGAAA GTTTG-TTGA
    4 logv  -ATTATAGTTTACGC TAGCTTCGGATCGGG CA---TCGATGT-AC TTAACATGGACGCTA ATCTTATCG-

    5 CpGV  -TTTGTGGTGTACGT GAGTTTTGGATCGAT GC---TCGACGC-CA CCACCATGGACCACC GTGCCCTAC-
    6 AFgv  GAATGTGGTGTACGT GAGCTTTGGTTCTAG CA---TAGACGC-GA TGGAAATGGACAGTG GGTTGATTG-
    7 CFNPV -G-AGCAATCTACGT GAGCTTCGGCTCCAG CA---TCGACAC-CA ACTCTATTCACGCCG AATT--TATA
    8 MbNPV -GGCGCTATTTACGT CAGTTTCGGTTCGGC AA---TATCCTC-CG AAGATATGGAGCCAG AGTTTATAG-

    9 ClGV  TGTAGGTATCTCAGT TCGGTGTAGGTCGTT CG--CTCCAAGCTGG GCTGTGTGCACGAAC CCCCCGTT--
```

```
Page 14.1
          1171       1185 1186       1200 1201       1215 1216       1230 1231
    1 AhGV  T-CAGAAAATGGTTA CCGCAGAGGAATATT TTG-AAGAACCCTCG TGTCAAGCTGTTCAT AACACAAGG
    2 AoGV  T-CAGAAAATGGTTA CCGCAGAGGAATATT TTG-AAGAACCCTCG TGTCAAGCTGTTCAT AACACAAGG
    3 CfGV  C-TAGAAATTGGTTT CCTCAAAGAGATATT TTA-AATCATCCCAA TGTAAAATTATTTAT AACACAAGG
    4 logv  CGCAGTCA-TGGTTC CCGCAACGAGACGTT TTA-AATCACCCGCA CATAAAAGTTTTCAT CACGCAGGG

    5 CpGV  C-TCGTGAATGGTTT CCGCAGAGAGCGCTG TTG-AACAGTGGTGT GGTGAAGTTGTTTGT GACGCAGGG
    6 AFgv  T-TAGGGAATGGTTT CCGCAAAGAGACATA TTG-AATCACGCTAA TGTTAAGCTGTTTAT TACTCAGGG
    7 CFNPV CACA-AAAGTGGTTT GACCAGCGCGCGGTG CTG-CATCACAAAAA AGTGGTGGCGTTCGT CATGCAAGC
    8 MbNPV TACAGTCG-TGGTTC GAACAATACAACCTG TTG-CATCATAAAAA CGTTCGTGCTTTTGT CACCCAAGG

    9 ClGV  C--A-----GGATTA GCAGAGCGAGGTATG TAGGCGGTGCTACAG AGTTCTTGAAGTGGT GGCCTAACT

Page 15.1
          1261       1275 1276       1290 1291       1305 1306       1320 1321
    1 AhGV  AGCGGTAGATAATGA AATACCTATGATTGT TGTGCCTTTGATGGG TGATCAATTTTTAAA CGCTCGCAA
    2 AoGV  AGCGGTAGATAATGA AATACCTATGATTGT TGTGCCTTTGATGGG TGATCAATTTTTAAA CGCTCGCAA
    3 CfGV  AGCTGTCGATAGCGA AATACCGCTTATTTG TATTCCAATGGTTGG GGATCAATTTGTCAA TTGTCGTAG
    4 logv  AGCAGTTAATAGCGG TGTTCCTATGATCGG GATTCCTATAATGGG CGATCAGTTTTATAA CGTGAGGCG

    5 CpGV  GGCGGTGGACGGCGG GGTGCCCATGCTGTG CATACCGATGGTGGG TGATCAATTTTTGAA TTGTGAACG
    6 AFgv  AGCGATAGATAGCTG TGTGCCCTTGCTGGG AATACCCATGGTGGG CGATCAGTTCTACAA CACGGGCCG
    7 CFNPV AGCACTGGAGTCGCG CGTACCTATGGTTTG TCTGCCCATGATGGG CGACCAATTTCACCA CGCGCGCAA
    8 MbNPV AGCAGTCGAAGCGAT CGTGCCGATGGTCGG CATGCCAATGATGGG CGATCAAGCCTACAA TATGAATAA

    9 ClGV  AGTATTTGGTATCTG CGCTCTGCTGAAGCC AGTTACCTTC---GG AAAAAGAGTTGGTAG CTCTTGATC
```

5.3.2.3) The identification of the *egt* gene in the ClGV-SA genome

The *egt* gene was successfully labelled with ^{32}P, using the oligonucleotide primed synthesis method. Medium stringency hybridisation of the probe to a Southern blot of restricted ClGV-SA and ClGV-CV3 DNA yielded a series of autoradiographic bands, corresponding to fragments in each RE profile (Fig. 40). The *egt* gene hybridised to fragment C in the *Bam*H1 digest, fragment A in the *Eco*R1 digest, fragment A in the *Xho*1 digest, fragment A in the *Kpn*1 digest and fragment B in the *Sac*1 digest, of the South African isolate. In the CV3 isolate, the probe hybridised to fragment A in the *Eco*R1 digest, fragment J & H in the *Bam*H1 digest, fragment A in the *Kpn*1 digest, fragment B in the *Sac*1 digest and fragment C in the *Xho*1 digest. Smith & Goodale (1998) state that an ORF 909 separates the *egt* and granulin genes in ClGV-CV3, with *egt* located to the left of ORF 909. The *egt* gene in the ClGV-SA isolates lies within the same fragments as the granulin gene (in most cases) (see Fig. 41). This gene could possibly be adjacent to granulin, as in ClGV-CV3; but this could not be conclusively shown.



1 – SA/*Bam*H1 digest
2 - CV3/B*am*H1 digest
3 – SA/*Eco*R1 digest
4 – CV3/*Eco*R1 digest
5 – SA/*Kpn*1 digest
6 – CV3/*Kpn*1 digest
7 – SA/*Sac*1 digest
8 – CV3/*Sac*1 digest
9 – SA/*Xho*1 digest
10 – CV3/ *Xho*1 digest
11 – λ/*Hind* III marker
12- 1 kb ladder

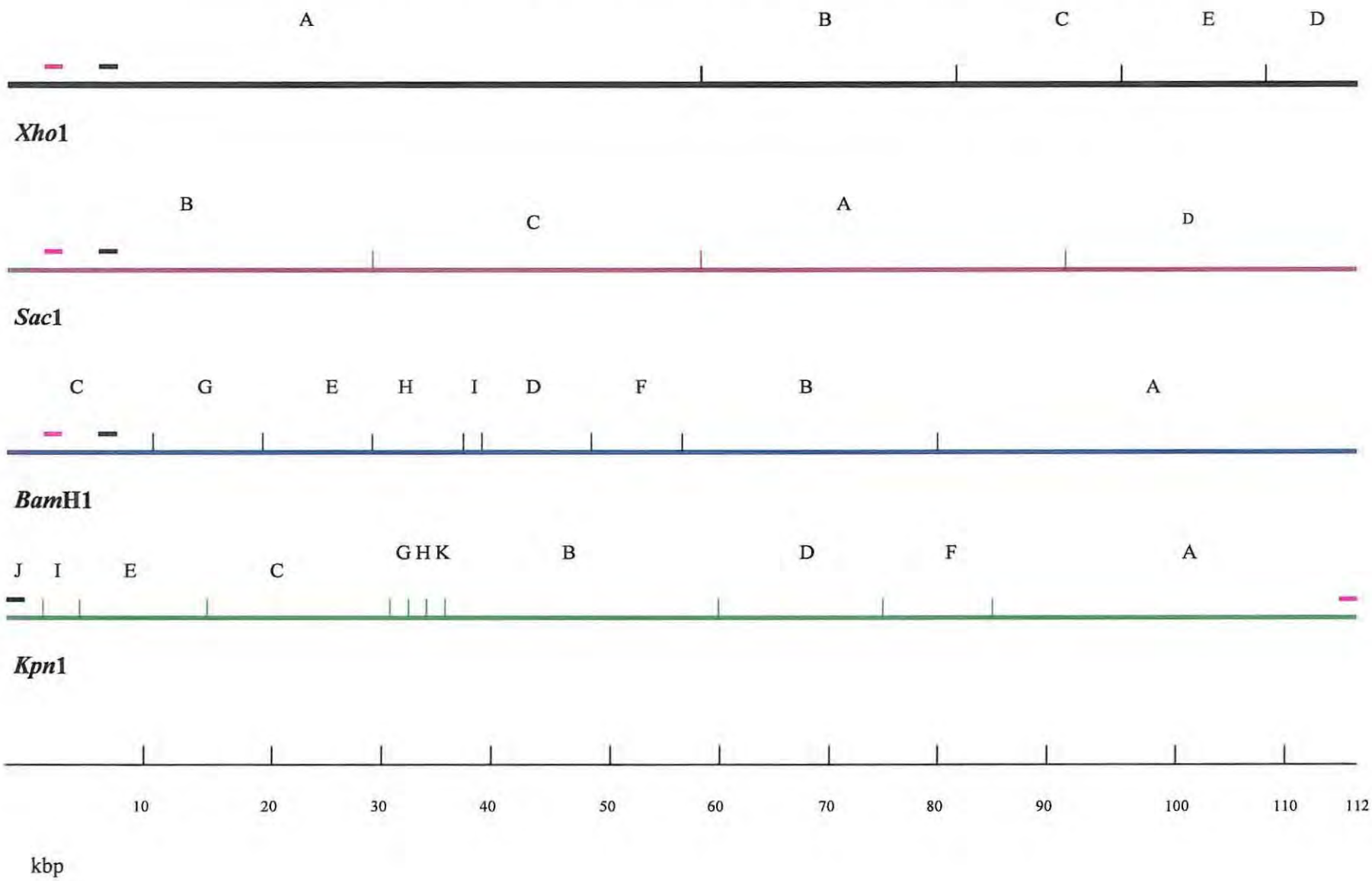
Figure 40: The position of the *egt* gene in the ClGV-SA genome. The ^{32}P-labelled probe hybridised to a particular fragment or fragments in each RE digest. A photograph of the corresponding RE digests of ClGV-SA DNA is visible beneath the X-ray.

5.4) DISCUSSION

5.4.1) Granulin

The granulin gene was successfully amplified from the ClGV genome and its position in the genome determined. A zero point for the physical maps for *Bam*H1, *Eco*R1, *Kpn*1, *Sac*1 and *Xho*1 was thus defined as the smallest fragment containing the granulin gene, e.g. fragment C in the *Bam*H1 digest was the smallest fragment containing the gene and was thus assigned as the zero point of the map (see Fig. 41). The gene was also sequenced and its sequence compared to the ClGV-CV3 granulin sequence, illustrating a great degree of homology between the granulin genes from

Figure 41: The position of the granulin and *egt* genes in the ClGV-SA genome. Pink = *egt*; black = granulin



both isolates. This result was expected since the granulin gene is reported to be highly conserved.

5.4.2) *egt*

After polyhedrin/granulin, more *egt* genes have been sequenced than any other baculoviral gene (Clarke *et al*, 1996). PCR amplification using the primers designed for *egt*, yielded a gene product. To investigate the position of the gene in the genome, a probe of the gene was made by labelling it with ^{32}P. The probe hybridised to different fragments in the Southern blot of the restricted ClGV-SA and ClGV-CV3 DNA; thus revealing the position of the gene within the ClGV genome (Fig. 41). A preliminary sequence of the *egt* gene was established. An alignment of this sequence with six other granulovirus *egt* genes revealed that the gene isolated is *egt*. But there exists many areas of non-alignment, seen in the translation of the sequence. Therefore *egt* needs to be sequenced in the opposite direction with suitable overlap in order to produce a complete sequence of the gene.

CHAPTER SIX: DETECTION ASSAYS FOR *Cryptophlebia leucotreta* GRANULOVIRUS IN FALSE CODLING MOTH LARVAE

6.1) INTRODUCTION

Baculoviruses are being used as agents to control insect pests of crops over large areas of agricultural and forest lands, in some instances on a commercial basis (Summers *et al*, 1975). Consequently there is a need for rapid and specific diagnostic methods to detect and monitor baculoviruses in ecological and epidemiological studies (Kelly *et al*, 1978). False codling moth larvae are responsible for extensive damage to citrus, rendering infested fruit no longer suitable for consumption, while light damage is sufficient for rejection on the export market.

False codling moth (FCM) populations undergo periodic outbreaks to very high densities that result in widespread fruit infestation, followed by epizootics of ClGV which reduce larval populations to low levels (Keating *et al,* 1989). An investigation was conducted into the occurrence of ClGV in the citrus orchards during a fruiting season. A detection assay would be useful to determine the virus incidence in orchards and to investigate whether the incidences of virus could be correlated to other factors affecting the larvae. Measuring virus incidence has previously been a time consuming and labour intensive process, using microscopic examinations of cadavers to confirm the presence of virus. The development of a quick and relatively inexpensive screening assay is thus required. One such technique is to use radiolabelled viral DNA probes in a hybridisation assay to detect the presence of viral DNA in larval extracts. Polymerase chain reaction (PCR) is another possible technique.

6.2) MATERIALS AND METHODS

Larvae were collected from two citrus orchards during two seasons. During the 1999-2000 season larvae were collected weekly from the Potgieter and Coetzee orchards, in the

Sundays river valley in the Eastern Cape. During the 2000-2001 season larvae were collected from infested fruit from the Ferriera and Rietfontein orchards, in the same region. The field-collected larvae were screened for ClGV using the dot blot assays and occasionally PCR.

6.2.1) Dot blotting of larval DNA

Dot blots permit rapid virus detection. Samples were applied directly onto nitrocellulose membranes under a vacuum through a multi-well dot blot filtration manifold. Samples were then fixed onto the filter. Hybridisation of a probe was achieved in the same way as with Southern blots and detection was by autoradiography. The technique used for dot blotting of samples was as follows:

Each larva was assessed for its larval stage (1st, 2nd instars), the date of its collection and area of origination (Coetzee or Potgieter orchard). 20 μl of 10 % SDS and 200 μl of 0.2 M sodium hydroxide was added to each larva in an eppendorf tube. The larva was squashed and then centrifuged at maximum speed in a bench top microfuge for 2 minutes. The supernatant was pipetted into a clean eppendorf. An equal volume of Tris-buffered phenol (pH 8) was added to the supernatant, which was mixed and centrifuged at maximum speed for 2 minutes. The upper aqueous phase was extracted into a clean eppendorf. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the tube was mixed and centrifuged as before. The upper aqueous phase was extracted again. The sample was then boiled for 2 minutes and cooled on ice briefly. 500 μl of ice-cold water was added. Samples were then ready for application onto the blotting manifold. Positive controls, which included larvae infected with ClGV (treated with the same process) and a ClGV DNA dilution series (to enable the concentration of virus present to be estimated), were also applied to the membranes.

Individual larvae were blotted onto nitrocellulose from the Potgieter & Coetzee orchards and the Rietfontein & Ferreira orchards. Larvae from the Potgieter & Coetzee orchards were collected from 22 December 1999 to 11 May 2000 at regular intervals. A total of

412 larvae were collected from both these orchards. On average 60 larvae were dot blotted onto a single piece of nitrocellulose. During the 2000 to 2001 season 259 larvae were collected (from 19th of December 2000 to the 9th of May 2001), from the Ferreira and Rietfontein orchards.

6.2.2) Assembly of the blotting manifold

The two halves of the blotting apparatus were separated. A piece of nitrocellulose membrane (the size of the blotting manifold) was hydrated in distilled water and then soaked briefly in 20 ml of 15 x SSC buffer. A piece of Whatmans 3 MM paper (the size of the blotting manifold) was placed on the bottom half of the manifold with the nitrocellulose membrane placed on the top half. The two halves were clamped together. The samples of larval DNA were applied to the wells with suction. Once the samples had passed through, 100 μl of 6 x SSC buffer was added to each well and passed through by suction. The manifold was then dismantled and the nitrocellulose membrane allowed to air-dry. The viral DNA was fixed into position was exposing the blot to UV light for 5 minutes on the transilluminator. Samples were blotted in every alternate row on the manifold because larval DNA often leaked into adjacent wells.

6.2.3) Development of probes

Two techniques were used for the labelling of probes. For radioactive labelling, ClGV DNA was fragmented and labelled with ^{32}P using nick translation. For the non-radioactive labelling, the granulin gene had DIG incorporated using random priming.

6.2.3.1) Labelling of ClGV with ^{32}P

Nucleic acid probes commonly have radiolabelled ^{32}P incorporated into the molecule, which permits its detection by autoradiographic techniques. Nick translation was used for the incorporation of the ^{32}P label into the ClGV DNA probe.

10 μl of ClGV DNA (approximately 50 μg) was made up to 200 μl with TE buffer (10 mM, pH 8) and the DNA was fragmented by passing through a 1 ml syringe and 26 G

needle. The DNA was then preciptated with two volumes of 95 % ethanol and 1/10th volume 3 M sodium acetate at –20°C for 20 minutes. The precipitant was centrifuged for 5 minutes at maximum speed and the DNA pellet resuspended in 20 µl TE buffer (10 mM, pH 8). 4 µl of DNA was electrophoresed on a 1 % agarose gel to ensure that fragmentation had occurred.

NICK TRANSLATION

In this process, DNA polymerase I adds nucleotides to the 3' hydroxyl terminus that is created when one strand of the dsDNA molecule is nicked. The enzyme, by virtue of its 5'-3' exonucleolytic activity, removes nucleotides from the 5' side of the nick. The simultaneous addition of nucleotides on the 3' end and removal of nucleotides from the 5' end results in the movement of the nick along the DNA. Existing nucleotides are replaced with highly radioactive ones, ensuring that the probe was radiolabelled (Sambrook *et al*, 1989).

The following mix was made up:

5.0 µl ClGV DNA
2.5 µl 10 x nick translation buffer
1.0 µl unlabelled dNTP's (except dCTP) (10 mM)
3.0 µl 3000 Ci/mmol [^{32}P dCTP] (50 µCi)
10.0 µl dd water
21.5 µl

2.5 µl of DNA Polymerase I (10 u/µl) was added and mixed. The reaction was incubated for 1 hour at 16°C and terminated with 1 µl of 0.2 M EDTA (pH 8). The unincorporated nucleotides were removed using the Quick spin columns (Roche). Refer to Appendix 5 for the protocol used.

6.2.3.2) Measurement of radioactivity

Two Whatman GF/A glass fiber filters (25 mm in diameter) were impaled onto a polystyrene support. 2 µl of the probe was spotted onto each filter, which were allowed to dry. One filter remained unwashed. The other filter was washed three times in 200 ml of

5 % TCA/sodium pyrophosphate for 2 minutes with swirling. It was then transferred to 70 % ethanol, where it was incubated briefly. Both filters were dried for 2 hours. They were then transferred to vials containing 5 ml of scintillation fluid and the counts per minute determined using the liquid scintillation counter.

6.2.3.3) Non-radioactive labeling of probes

A safer technique for the labelling of probes was using non-radioactive DIG (Roche). The granulin gene was isolated using PCR and labelled with DIG (Chapter 5). The protocol used in the labelling and detection of DIG probes was discussed in Chapter 4.

6.2.4) Hybridisation and detection

For the probes labeled with radioactive ^{32}P, hybridization buffer was warmed to 42°C. Refer to Appendix 5 for the solutions used. The hybridization buffer contained Denhardts reagent that blocked nonspecific attachment of the probe to the filter and dextran sulphate, which accelerated the rate of hybridization. 300 μl of sonicated calf thymus was added to 500 μl of double distilled water (final concentration of 100 μg/ml) and was boiled for 5 minutes and then cooled on ice for 10 minutes. The denatured DNA was added to 15 ml of pre-hybridization buffer, in which the dot blots were incubated for 2 hours at 42°C with agitation. The labelled probe was boiled for 5 minutes and cooled on ice for 10 minutes. Thereafter it was added to 15 ml of hybridization buffer and the blots were incubated in this buffer with probe overnight at 42°C, with agitation. The blots were then washed briefly in 2 x SSC, 0.1 % SDS to drain off the radioactivity. The next wash was at room temperature for 15 minutes in 2 x SSC, 0.1 % SDS. Two washes at medium stringency followed with both washes done twice. The first was in 4 x SSC, 0.1 % SDS for 15 minutes at room temperature. The next wash was in 0.5 x SSC, 0.1 % SDS for 30 minutes at 65°C. The blots were pressed dry between sheets of Whatman paper. It was then placed into an autoradiography cassette under X-ray film and incubated at –70°C. The period of incubation depended on how radioactive the probe was. The X-ray was later developed.

For the non-radioactive system, the hybridization and detection of probes labelled with DIG were performed as in Chapter 4. Detection was also via autoradiography.

6.2.5) Polymerase chain reaction (PCR)

PCR is generally used to amplify a segment of DNA that lies between two regions of known sequence. It results in an efficient exponential amplification of the target sequence (Sambrook *et al*, 1989). To determine if the field-collected larvae were infected with ClGV, PCR was used to amplify the granulin gene. It was assumed that if a larva was infected with ClGV, the granulin gene would be amplified from the virus. PCR is a sensitive technique that is able to detect small quantities of DNA; therefore it was chosen for use in the detection of virus from field-collected larvae.

Viral DNA was extracted from individual larva using a technique adapted from Moraes *et al* (1998). Each larva was macerated in homogenization buffer (see Appendix 5 for solutions used) and centrifuged at 3000 g for 2 minutes to remove insect debris. The pellet was resuspended in 500 μl 1 M Tris-HCl (pH 8) and centrifuged at 10 333 g for 10 minutes. The pellet was resuspended again in 500 μl 1 M Tris-HCl (pH 8) and treated in 1/3 of the total volume of an alkali solution to disrupt the granules. The alkali-released virus was centrifuged at 10 333 g for 10 minutes and resuspended in 500 μl 1 M Tris-HCl for neutralization purposes. These crude DNA preparations were diluted 100 times and used as templates for PCR amplification with the granulin primers. The PCR cycle on the PCR*Sprint* (Hybaid), used for the amplification of granulin is discussed in Appendix 4.

6.3) RESULTS

6.3.1) Labelling of probes

6.3.1.1) Radiolabelling with ^{32}P

To determine if the probe had successfully incorporated the ^{32}P label, the counts per minute (cpm) were determined using the liquid scintillation counter. The cpm of the probe is shown in Table 10, indicating that the probe had incorporated the ^{32}P. The counts obtained from the washed probe were high and not significantly lower than that of the

Table 10: The counts per minute determined from the ^{32}P labelled probe.

	VOLUME OF PROBE	COUNTS PER MINUTE
UNWASHED	2 µl	466840.00 cpm
WASHED	2 µl	389648.00 cpm

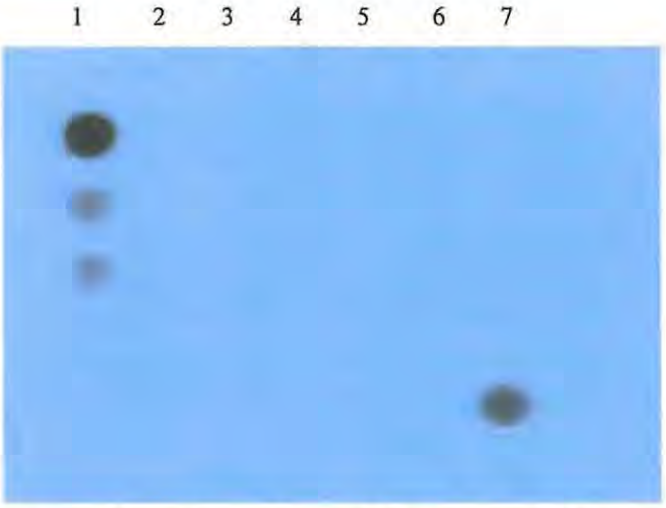
6.3.1.2) Labelling of probes with a non-radioactive label

The granulin gene was labeled with DIG. Since the total volume of the probe was required for the hybridization and detection, it was not determined if labelling was successful.

6.3.2) The detection of ClGV from the dot blots

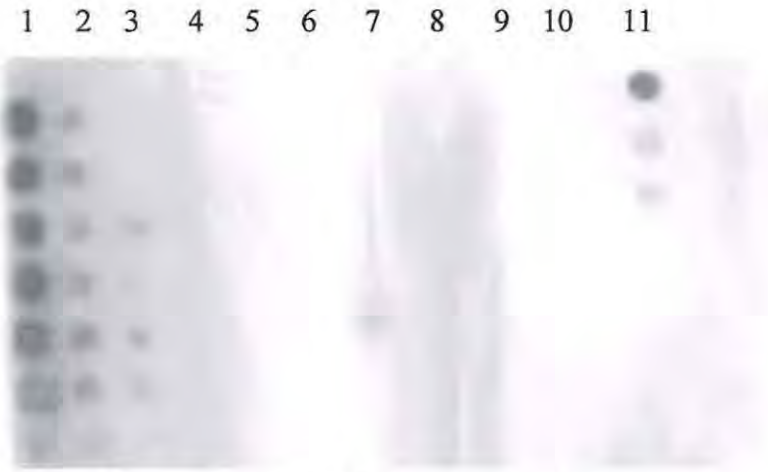
Signals produced on X-ray film indicated that the sample contained ClGV DNA. The results of probing two such dots blots with a radioactive and a non-radioactive probe are presented in Figs. 42 & 43. In Fig. 42, the binding to a ClGV DNA dilution series, from 8 µg/ml to 2 µg/ml, was shown in row 1. Concentrations of 8 µg/ml to 2 µg/ml of ClGV DNA were detectable on the blot. In row 7 a positive signal for a field-collected larva from the Potgieter orchard was detected. No other signals were produced indicating that all other larvae were virus free. The DIG labelled probe was used for this blot. In Fig.43, row 1 contains 7 ClGV infected larvae that all produced positive signals. Viral DNA appeared to have spilt over into the adjacent lanes. The DNA dilution series as above was included in row 11. One field collected larva (row 7) produced positive signal when probed with the ^{32}P labelled probe. The larva was collected from the Rietfontein orchard on the 20th of March 2001.

Both probes were successful in binding to the target DNA since the diseased larvae and ClGV DNA both produced positive signals in every blot. Smeared signals occurred on certain blots due to the leakage of larval DNA into neighbouring wells. The addition of an extra layer of Whatman paper on the bottom half of the blotting manifold generally limited the amount of leakage. Each dot blot was assessed for the presence of ClGV and the results are presented in 6.3.5.



1 – ClGV DNA dilution series
2 – Coetzee Orchard – 22/12/99
2 – Potgieter Orchard – 13/01/00
3 – Potgieter Orchard – 19/01/00
4 – Potgieter Orchard – 02/02/00
5 – Potgieter Orchard – 16/03/00
6 – Potgieter Orchard – 23/03/00
7 - Potgieter Orchard – 24/02/00

Figure 42: Probing of a dot blot with a DIG-labelled ClGV probe.



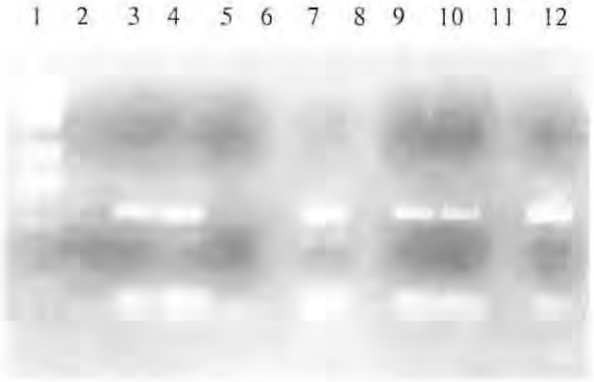
1 – ClGV infected larvae
3 – Rietfontein – 31/01/01
5 – Rietfontein – 14/02/01
7 – Rietfontein – 20/03 01
9 – Rietfontein – 24/04/01
11 – ClGV DNA dilution series

Figure 43: Probing of a dot blot with a ^{32}P labelled probe.

6.3.3) PCR detection of ClGV

Single larvae were selected from the Rietfontein and Ferreira orchards and ClGV was detected using PCR. The granulin primers were used and if the larvae were infected with ClGV, a PCR product of 747 bp would be produced. Fig. 44 presents some of the results obtained. A positive control (granulin PCR product) was included in lane 12. There were granulin PCR products present on the 1 % agarose gels (lanes 3, 4, 7, 9 and 10), indicating that those larvae were infected with ClGV. The results of the PCR were

ClGV, a PCR product of 747 bp would be produced. Fig. 44 presents some of the results obtained. A positive control (granulin PCR product) was included in lane 12. There were granulin PCR products present on the 1 % agarose gels (lanes 3, 4, 7, 9 and 10), indicating that those larvae were infected with ClGV. The results of the PCR were correlated with the results of the dot blotting to determine if the incidence of virus detection was the same using both techniques.



1 – λ/*Bst*E II marker
2 – Ferreira – 20/02/01
3 – Rietfontein – 10/04/01
4 – Rietfontein – 18/04/01
5 – Rietfontein – 20/02/01
6 – Rietfontein – 28/02/01
7 – Rietfontien – 14/02/01
8 – Ferreira – 27/03/01
9 – Rietfontein – 24/04/01
10 – Reitfontein – 7/05/01
11 – Rietfontein – 20/02/01
12 – control - granulin

Figure 44: Detection of ClGV from field collected larvae using PCR.

6.3.4) The incidence of ClGV in citrus orchards

The data obtained from the dot blotting and PCR reactions were used to determine the incidences of ClGV in the orchards, as well as to indicate any trends in infection. The results from the Potgieter and Coetzee orchards are presented in tables below. The incidence of ClGV was monitored from December 1999 to May 2000 and the number and percentage of larvae that were infected with virus were recorded in Table 11.

Table 11: Number of infected larvae detected from each collection date.

DATE	22/12	12/01	13/01	19/01	26/01	02/02	09/02	16/02	24/02
POTGIETER	2/6	3/6	0/0	0/6	1/4	0/0	2/4	0/0	1/5
%	33 %	50 %	0 %	0 %	25 %	0 %	50 %	0 %	20 %

COETZEE	0/0	2/10	0/0	2/10	3/7	11/22	2/33	0/60	3/49
%	0 %	20 %	0 %	20 %	43 %	50 %	6 %	0 %	6 %

DATE	02/03	16/03	23/03	31/03	06/04	13/04	20/04	04/05	11/05
POTGIETER	1/3	0/4	0	0	0	0/19	0	0/13	0/7
%	33 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %
COETZEE	0/35	0/9	3/9	0/16	0/17	2/36	0/16	3/44	0
%	0 %	0 %	33 %	0 %	0 %	6 %	0 %	6 %	0 %

The instars that were infected and the percentage of infection from these orchards are tabulated in Tables 12 & 13 and illustrated in Fig. 45.

Table 12: Infectivity in different instars

	1st instar	2nd instar	3rd instar	4th instar	5th instar
Potgieter	1/10	1/4	3/17	0/5	1/11
Coetzee	0/28	5/63	13/96	2/27	5/91

Table 13: Percentage infectivity in different instars

	1st instar	2nd instar	3rd instar	4th instar	5th instar
Potgieter	10 %	25 %	18 %	0 %	9 %
Coetzee	0 %	8 %	14 %	7 %	5 %

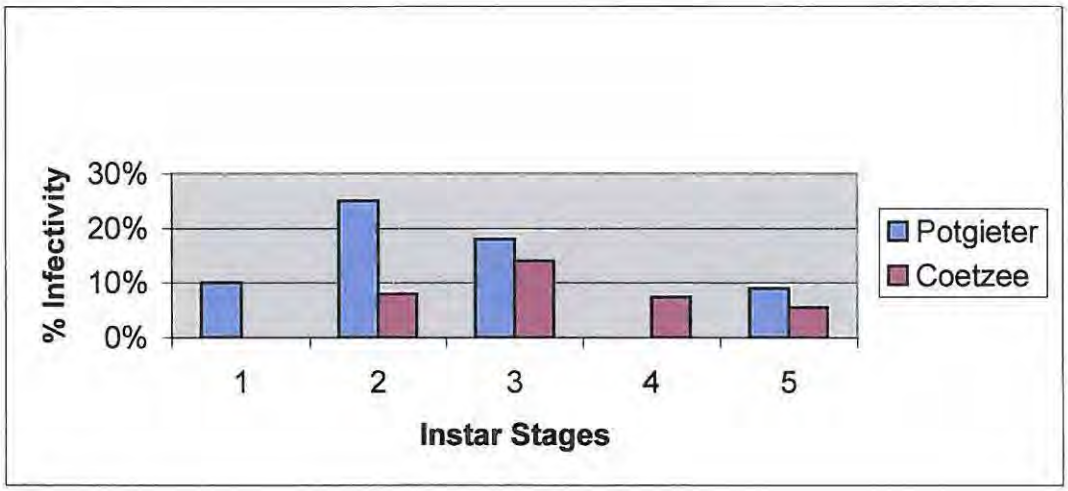


Figure 45: The percentage infectivity of different instars

A ClGV DNA dilution series was included on the blots. This assisted in determining the concentration of virus present in the field-collected larvae. These results are presented in Table 14 and Fig. 46.

Table 14: The concentrations of ClGV detected in field-collected larvae.

	VIRUS CONC.	8 µg/ml	4 µg/ml	2 µg/ml
POTGIETER	% OF LARVAE INFECTED	10 %	30 %	60 %
	INSTAR INFECTED	$3^{rd} - 5^{th}$ instar	$3^{rd} - 4^{th}$ instar	$2^{nd} - 3^{rd}$ instar
COETZEE	% OF LARVAE INFECTED	21 %	68 %	7 %
	INSTAR INFECTED	$3^{rd} - 5^{th}$ instar	$3^{rd} - 4^{th}$ instar	$2^{nd} - 3^{rd}$ instar

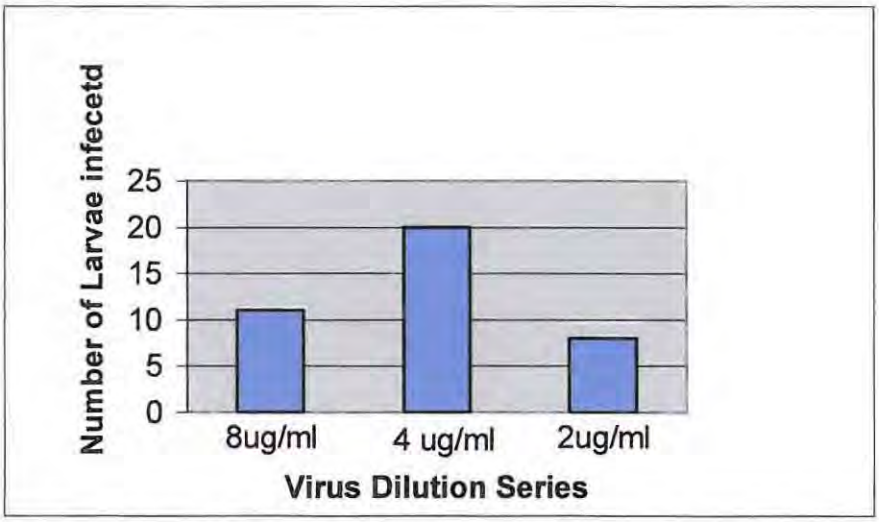


Figure 46: Number of infected larvae containing virus at the indicated concentration.

Using the information from the tables and graphs, deductions can be made about the incidence of ClGV in these orchards. These deductions will be explained in section 6.4. A smaller study investigating virus incidence in the Rietfontein and Ferreira orchards during December 2000 to May 2001 was conducted, with the results presented in Tables 15 and 16 and Fig. 47. A total number of 177 larvae were collected from stung fruit in the Rietfontein orchard and 82 from the Ferreira orchard. The incidences of ClGV are presented in Table 15.

Table 15: Number of infected larvae in the Rietfontein and Ferreira Orchards

DATE	**19/12**	**25/01**	**01/01**	**02/01**	**09/01**	**16/01**	**17/01**	**23/01**	**31/01**
RIETFONT .	-	-	-	-	-	-	-	-	-
%	-	-	-	-	-	-	-	-	
FERREIRA	0/3	0/17	0/4	0/16	0/16	0/2	0/8	0/4	0/14
%	0 %	0 %	0 %	0 %	0 %	0 %	0%	0 %	0 %

RIETFONT.	0/3	0/6	0/13	0/13	0/4	0/3	1/4	0/2	1/2
%	0 %	0 %	0 %	0 %	0 %	0 %	25 %	0 %	50 %
FERREIRA	0/1	0/5	0/4	0/12	0/3	0/2	0/4	0/1	0/1
%	0 %	0 %	0 %	0 %	0 %	0 %	0%	0 %	0 %

DATE	**07/04**	**10/04**	**18/04**	**24/04**	**02/05**	**09/05**
RIETFONT.	0/4	1/10	2/5	1/6	-	4/8
%	0 %	10 %	20 %	16 %	-	50 %
FERREIRA	0/3	0/2	0/6	-	0/3	0/8
%	0 %	0 %	0 %	-	0 %	0 %

(-): No larvae were collected on that date.

The frequency of larvae infected with ClGV in the Rietfontein orchard was 12 % and the Ferreira orchard had a 0 % infection. The different instars that were infected in the Rietfontein orchard were as follows:

Table 16: The infection frequency of instars in the Rietfontein orchard

	1st INSTAR	2nd INSTAR	3rd INSTAR	4th INSTAR	5th INSTAR
RIETFON.	1/11	6/23	0/17	0/9	2/25
%	9 %	26 %	0 %	0 %	8 %

Fig. 47 illustrates the percentage of infection in the different instars in the Rietfontein orchard.

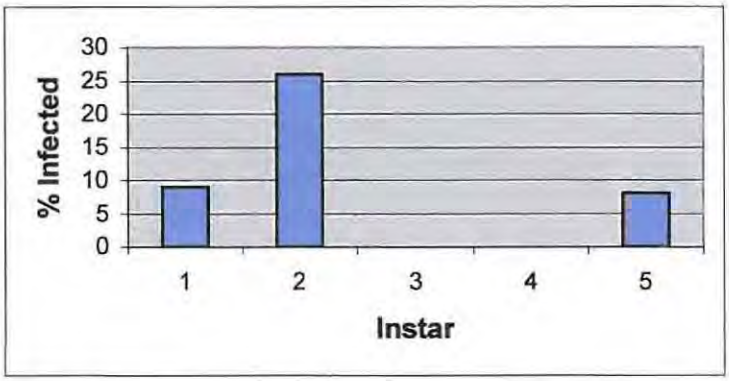


Figure 47: The percentage of each larval instar infected with ClGV

Figs. 48 and 49 illustrate the percentage of fruit covered with eggs, and the percentage of FCM-induced fruit drop per tree, from the Rietfontein orchard. The release and transition areas were two different sectors of the orchard. Numbers 1-14 are the collection dates of larvae from the Rietfontein orchard presented in Table 15 (06/02 to 09/05, excluding 02/05). This data could be correlated to the incidence of ClGV detected in the orchard, and a trend of ClGV occurrence established. Deductions regarding ClGV incidence is discussed in 6.4.

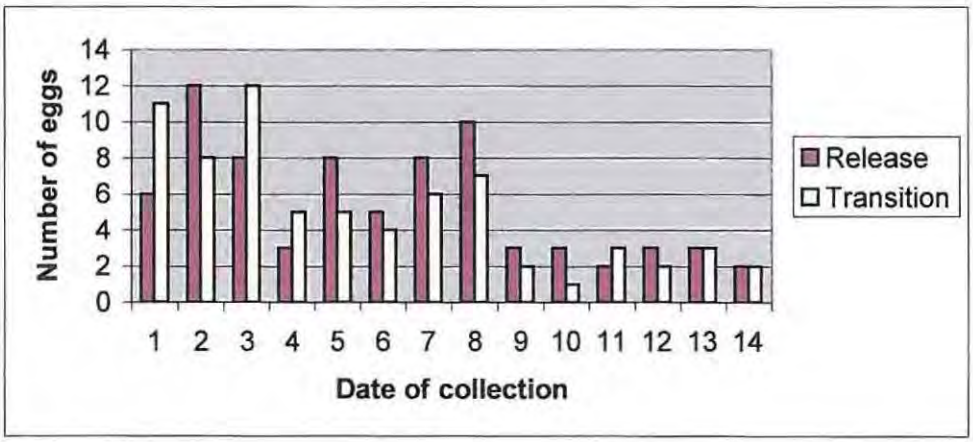


Figure 48: The number of fruit covered with FCM eggs in the Rietfontein orchard.

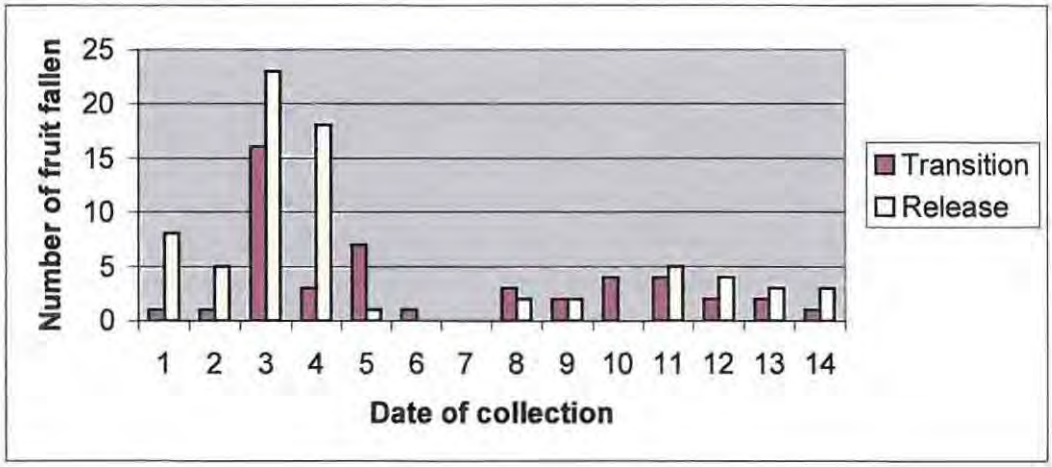


Figure 49: The number of fruit that fell due to FCM infestation

6.4) DISCUSSION

These experiments demonstrate the feasibility of using labelled DNA probes in hybridization assays to determine virus incidence in field-collected larvae. The dot blot technique was advantageous since it was not labour intensive, many samples were probed for ClGV simultaneously and it was quick. The amount of virus in each larva could be estimated by comparison with standard amounts of purified viral DNA. This technique could significantly improve the accuracy of estimation of virus incidence in field populations. It now may be possible to test larvae to determine their cause of death. If death was due to ClGV infection, then highly specific DNA probes would be able to detect this. This technique thus reduces the uncertainty and ambiguity of microscopic diagnosis. It also may be possible to adapt the technique to detect viral DNA in adults, eggs, pupae and environmental samples (soil or leaves). The use of non-radioactive probes is favoured since these probes are far safer and more stable.

PCR is a highly sensitive technique and is able to detect smaller quantities of ClGV. It would be an ideal technique to use for the screening of field populations of larvae, but due to the high costs involved dot blotting proved to be more feasible since large numbers of larvae are processed in bulk at a cheaper cost.

From the results of the field studies, it was determined that ClGV was detected in both the Potgieter and Coetzee orchards. The virus infected the larvae throughout the season, but most infections occurred in January, February and March 2000. In the Coetzee orchard, significant detection of ClGV occurred in January and February with 50 % of the larvae screened on the 2nd of February infected with ClGV. The incidence of ClGV dropped in March, April and May. Most of the infected larvae were in the 3rd instar stage and most were infected with approximately 4 μg virus/ml of insect extract. In the Potgieter orchard, ClGV was detected from late December to early March, with the incidence of virus decreasing from March to May. Mostly 2nd and 3rd instars were infected at a level of approximately 2 μg virus/ml insect extract.

A significant result was obtained for the Ferriera orchard in that no ClGV was detected throughout the season. In the Rietfontein orchard, ClGV was detected in April and May. The trend was that little to no infection occurred early in the season with an increase in virus incidence later in the season. Mostly 2nd instars were infected, with no 3rd and 4th instars showing any infection. The results of the PCR detection confirmed the incidence of ClGV in both these orchards. Larvae from the Ferreira orchard that were screened using PCR also produced no PCR product. This confirmed the dot blotting results, that no ClGV was detectable in that orchard. Larvae from the Rietfontein orchard that were screened using PCR, confirmed that the incidence of ClGV was high during April and May and low during the rest of the season.

In order to determine any trends in the orchards with regard to the incidence of virus, the number of moths present, the percentage of fruit with eggs and the percentage of fruit infested with larvae, were noted in the Coetzee and Potgieter orchards (S Moore, personal communication). By correlating this information with the incidence of ClGV, it was determined that the infestation of fruit with larvae and eggs and the occurrence of adult moths declined significantly when the incidence of ClGV increased. When ClGV occurrence declined, there were more adult moths present, more FCM eggs were laid on fruit and the larvae infested a higher percentage of fruit. This study enabled the incidence

of virus to be monitored throughout the season. Significant deductions can be made from these results:

- When the insect population size grew, more eggs covered each fruit, more fruit became infested and increasing numbers of fruit fell from the trees, and the incidence of ClGV was low.
- However, those conditions led to an outbreak of ClGV. When the virus was detected in the orchard, the infestation of fruit, fruit fall and numbers of moths all declined.

In the Rietfontein orchard, the same trend was determined as in the Coetzee and Potgieter orchards. The percentage of fruit with eggs and FCM fruit drop are presented in Figs. 48 & 49. This data was correlated with the dot blotting and PCR results. ClGV was detected in the Rietfontein orchard from the 20th of March to the 9th of May. There were a large number of eggs on the fruit and many fruit had fallen from the trees prior to ClGV being detected in the orchard (6th Feb – 18 March). Following the occurrence of ClGV (20th of March to the 9th of May), both the number of eggs laid on the fruit and the number of fruit fallen from the trees, decreased for both the release and transition areas. The number of adult moths also declined once ClGV was detected in the orchard.

Since infestation of fruit by this pest is minimal when ClGV is present, it can be concluded that ClGV would be an effective control agent against FCM.

CHAPTER SEVEN: FINAL DISCUSSIONS AND FUTURE PROSPECTS

7.1) THE SIZE OF THE *Cryptophlebia leucotreta* GRANULOVIRUS GENOME

Genome sizes for different members of the baculovirus family vary from 90 to 160 kbp. The wide variation in size of the baculovirus genomes indicates that some members may lack many of the genes present in other members of the family (Miller *et al*, 1997). The size of the genome of ClGV-SA was calculated by summating the molecular weights of each band produced in a restriction enzyme digest. The bands had to be separated such that each band could be visualised on an agarose gel, to ensure that all bands were accounted for. The size of each band was then calculated using the molecular weight markers and Program gel computer program. The size of the genome was estimated to be 112 kbp. Therefore the size of the ClGV-SA genome proved to be the same as the size of ClGV-CV3 (Cape Verde isolate), which was determined to be 112 kbp by Jehle & Fritsch (1992). The genome size of other granuloviruses ranges from 100 kbp (*Adoxophyes orana* GV) to as large as 125.6 kbp (*Cydia pomenella* GV). However the size of the ClGV-SA genome was found to be very similar to *Artogeia rapae* GV, which was calculated to 110 kbp. The genome sizes of the nucleopolyhedroviruses lie within the same range as the granuloviruses.

7.2) A COMPARISON BETWEEN THE SOUTH AFRICAN ISOLATE OF *Cryptophlebia leucotreta* GRANULOVIRUS WITH THE CAPE VERDE ISOLATE OF THE VIRUS

An investigation into the similarity or differences between the two isolates was conducted, by comparing their restriction enzyme profiles and physical maps. Viral DNA was digested, singly and in combination, with a variety of restriction enzymes. The profiles produced were then compared. It was found that the ClGV-SA and ClGV-CV3 profiles differed significantly for each restriction enzyme. Restriction enzyme sites that were present in the ClGV-CV3 were absent in the ClGV-SA. Likewise new sites were present in ClGV-SA. For example, the largest fragment in the *Eco*R1 digest of ClGV-CV3 is 17.0 kbp, but this fragment was absent in the ClGV-SA profile due to a site being lost on the genome. There are 4 fragments in the *Sac*1 digest of ClGV-SA while the ClGV-CV3 digest was found to have only 3

fragments. The *Bam*H1 digest of ClGV-SA was found to have 9 fragments, as opposed to the 11 fragments in the ClGV-CV3 digest with *Bam*H1. In the *Kpn*1 profile, fragments sized 22.2 kbp and 11.8 kbp were present in the ClGV-SA profile but absent in the ClGV-CV3 profile. These differences were distributed throughout the genome for all the restriction enzymes used. These results showed that the isolates were very different. Relatedness is correlated to geographical origin (Crook, 1985). The ClGV-SA isolate is therefore distinct when compared to the Cape Verde isolate.

Five physical maps were constructed for *Bam*H1, *Eco*R1, *Kpn*1, *Sac*1 and *Xho*1. These maps were aligned with the CV3 physical maps for the same enzymes (constructed by Jehle & Fritsch, 1992). The alignments revealed differences between the isolates. For example, in the *Xho*1 digest fragment A in ClGV-SA is 57.5 kbp. But in ClGV-CV3 fragments B, C and D make up fragment A of the South African isolate. This indicates that two *Xho*1 sites were lost in ClGV-SA to create the large fragment A. In the *Sac*1 digest of ClGV-CV3, there are only 3 fragments while in ClGV-SA there are 4 fragments. A new site was created in fragment A of ClGV-CV3, to produce fragments A and D in ClGV-SA. Restriction enzyme sites were lost and new sites created in ClGV-SA. These results confirmed the fact that the two isolates differ.

7.3) THE GRANULIN AND *egt* GENES

The granulin gene is the most widely sequenced gene among the granuloviruses. The gene was amplified successfully from the ClGV-SA genome, by PCR. Its position in the genome located. The gene was then used as the zero point for the construction of the physical maps. The gene was sequenced and this sequence compared to the granulin gene of the ClGV-CV3 isolate. Alignments of the two genes revealed that only six nucleotides differed between them. Their amino acid sequences were identical. This result confirms that the granulin gene is highly conserved.

The *egt* gene blocks the moulting of the insect, resulting in greater feeding and more crop damage. An *egt* strain of virus is reported to cause the insect to moult soon after infection, thus limiting crop damage and increasing the speed of kill of the virus (Crook *et al*, 1997). An *egt* negative genotype into which a foreign gene is inserted may be an attractive and feasible alternative for the genetic improvement of

baculoviral pesticides (Smith & Goodale, 1998). *egt*⁻ strains of virus have already been engineered for CpGV. This gene is not highly conserved. Therefore, to isolate the gene from the ClGV-SA genome, conserved regions from the alignment of other granulovirus *egt* sequences had to be determined for the design of primers for the amplification of the gene using PCR. The position of the gene was then located in the genome. The data suggests that the *egt* gene is located very close to the granulin gene, as in ClGV-CV3. The ClGV-SA *egt* gene was then sequenced and aligned with other granulovirus *egt* sequences. The alignments revealed considerable differences between the ClGV-SA sequence and the other *egt* sequences. The translation of the sequence produced many stop codons suggesting that it contained many errors. These results suggest that the sequence obtained is an incomplete one and further sequencing of the gene is required. However it can be deduced that the gene isolated is in fact *egt*.

7.4) THE DETECTION ASSAY FOR *Cryptophlebia leucotreta* GRANULOVIRUS

An investigation into the incidences of ClGV in natural populations of FCM larvae was conducted. The aim was to find a technique that could successfully detect ClGV in the field-collected larvae, and to determine if any trends regarding the incidence of virus in the orchards existed. Dot blotting of larvae proved to be feasible since many larvae could be blotted on a single blot using a quick and inexpensive technique. Nucleic acid probes, either radiolabelled or non-radiolabelled, were successful in detecting ClGV from collected larvae and in positive controls of infected larvae and ClGV DNA. Although both probes achieved the same outcome, the non-radiolabelled DIG probe was favoured due to its safety and stability. The PCR detection assay for ClGV proved to be a relatively quick detection system that was able to detect very small quantities of virus effectively. PCR is by far the more sensitive technique for the detection of ClGV. However, the cost of the enzymes used in PCR for the amplification of DNA is high, therefore this technique has proved to be very expensive. Dot blotting of samples and the probing with labelled probes has proved to be the most suitable technique for detecting virus. This method is inexpensive, allows the rapid screening of hundreds of samples and is not labour intensive.

From the study conducted in the two sets of orchards during two fruiting seasons, certain trends regarding the incidence of ClGV could be determined. The occurrence

of virus was greater at certain times during the season. When the larval populations grew, the number of infested fruit increased, as well as the number of larvae and moths. The increased numbers of moths were found to lay more eggs on fruit and as a result, more fruit fell from the trees. The incidence of ClGV in the orchard then became higher. Once ClGV was detected, the virus was able to limit the numbers of moths and larvae as well as the number of fruit infested. This trend was evident in both studies that were conducted. ClGV was detected in three of the four orchards screened, with the Ferreira orchard remaining virus-free. In the first study, it was found that most of the infected larvae were in the 3rd instar stage and most were infected with approximately 4 μg virus/ml of insect extract. In the Rietfontein orchard (second study), mostly 2nd instars were infected, with no 3rd and 4th instars showing any infection. It was also evident that ClGV was prevalent in the orchard towards the end of the season. The results of the PCR reactions confirmed these findings and that the Ferreira orchard was virus-free.

7.5) FUTURE WORK

Due to its safety and high specificity against its target host, ClGV offers an attractive alternative to chemical pesticides. The virus may soon be used on a commercial level. Field trials investigating the efficacy of ClGV and a suitable formulation of the virus, i.e. one that prevents the breakdown of the occlusion body by UV light, are required. Once the ability of ClGV to control natural larval populations is known, the virus could be further engineered for use commercially.

On a molecular level, the *egt* gene needs to be sequenced completely in both directions to produce a dependable sequence of the gene. A phylogenetic analysis of the gene with other granulovirus and nucleopolyhedrovirus *egt* genes can be established. Other genes, e.g. cathepsin, chitinase and enhancing, could be isolated and identified. Cathepsin and chitinase function together to facilitate host cuticle breakdown after death, ensuring the release of progeny virus into the environment for horizontal transmission (Miller *et al*, 1997). Enhancin facilitates virus-host cell fusion and the disruption of the peritrophic membrane, thus contributing to viral progeny (Miller *et al*, 1997). Manipulations of these genes could enhance the properties of the virus, enabling it to kill the larval host faster. An *egt*$^{-}$ strain of ClGV would be of great benefit, since the virus without a functional *egt* gene would cause larvae to moult,

thereby limiting the damage done to the crops. A cell culture of ClGV would therefore be required to enable these manipulations to be performed. Future work on the project could also include obtaining a genotypically pure strain of virus for manipulations. Baculoviruses often occur as a mixture of genotypes. It would be desirable to work with a cloned strain of a single genotype to eliminate variability. The establishing of a *C.leucotreta* cell culture would facilitate the study of ClGV and the subsequent engineering of ClGV as a suitable biological control agent.

APPENDIX 1: VIRUS PURIFICATION AND DNA EXTRACTION

1) Virus purification

0.1 % SDS (100 ml)

0.1 g SDS

100 ml water

80 % GLYCEROL (50 ml)

10 ml 0.1 % SDS

40 ml glycerol

30 % SDS (50 ml)

35 ml 0.1 % SDS

15 ml glycerol

2) DNA extractions

1 M Tris-HCl (pH 8)

Dissolve 121.1 g of tris base in 800 ml of distilled water. Adjust pH to 8 with HCl. Make up to 1 L and autoclave.

TRIS-BUFFERED PHENOL (pH 8)

Melt 100 g of phenol at 68°C and then add an equal volume of 0.5 M Tris-HCl (pH 8) at room temperature. Stir on magnetic stirrer for 15 minutes. When the two phases have separated, aspirate the upper aqueous phase. Add an equal volume of 0.1 M Tris-HCl (pH 8) to the phenol and stir. Extract the upper aqueous phase. Repeat the extraction procedure until the pH of the phenol is greater than 7.8. Add 0.1 x volume of 0.1 M Tris-HCl (pH 8) containing 200 μl of β-mercaptoethanol and store the phenol at 4°C in dark bottle. The phenol should be made in a fume hood and pH checked using pH paper.

CHLOROFORM:ISOAMYL ALCOHOL (24:1) (100 ml)

96 ml of chloroform

4 ml of isoamyl alcohol

95 % ETHANOL (100 ml)

95 ml of pure ethanol

5 ml of water

Store at –20 °C.

70 % ETHANOL (100 ml)

70 ml of pure ethanol

30 ml water

Store at –20°C.

10 % SDS (100 ml)

10 g SDS

100 ml water

Warm to 65°C to allow SDS to dissolve.

3 M SODIUM ACETATE (1 L)

408.1 g sodium acetate

800 ml water

Adjust the pH to 7 with dilute acetic acid. Make up to 1 L and sterilise by autoclaving.

1 M SODIUM CARBONATE (200 ml)

22 g sodium carbonate

200 ml water

Sterilise by autoclaving.

0.01 M EDTA (100 ml)

0.327 g EDTA

100 ml water

PROTEINASE K (25 mg/ml)

0.0025 g Proteinase K

1 ml of water

Store at –20°C.

RNase A

Dissolve 0.1 g of RNase A powder in 1 ml of water.

TE BUFFER (10 mM Tris/HCl, pH 8.0, 0.1 M EDTA) (1 L)

121.1 g tris base

2 ml 0.5 M EDTA (pH 8.0)

Make up to 1L with distilled water and adjust pH to 8.0 with concentrated HCl. Sterilise by autoclaving. (pH the tris to 8.0 before adding the EDTA and top up to 1L).

3) Genomic tips Q20 (Qiagen)

SOLUTION 1 (100 ml)

4.382 g sodium chloride (750 mM)

1.046 g Mops (4-morpholine-propanesulfonic acid) (50 mM)

pH to 7.0.

BUFFER QTB (50 ml)

2,19 g sodium chloride (750 mM)

0.523 g Mops (4-morpholine-propanesulfonic acid) (50 mM)

7.5 ml isoproponol (15 %)

0.075 ml Triton X-100 (0.15 %)

pH to 7.0.

BUFFER QC (50 ml)

2.922 g sodium chloride (1 M)

0.523 g Mops (4-morpholine-propanesulfonic acid) (50 mM)

7.5 ml isoproponol (15 %)

pH to 7.0.

BUFFER QF (50 ml)

7.5 ml isoproponol (15 %)

1.1 g tris base (50 mM)

3.65 g sodium chloride (1.25 M)

pH to 8.5 with HCl.

APPENDIX 2: THE ANALYSIS OF *Cryptophlebia leucotreta* GRANULOVIRUS DNA

1) DNA agarose gels

1 % AGAROSE GEL

0.7 g agarose powder

7 ml 10 x TAE

63 ml distilled water

Dissolve in microwave for 2 minutes. The gel is allowed to cool before 4 μl of 10-mg/ml ethidium bromide solution is added. Cast into a gel tray and allow setting.

0.7 % AGAROSE GEL

0.84 g agarose powder

12 ml 10 x TAE

108 ml distilled water

Dissolve in microwave for 2 minutes. The gel is allowed to cool and then cast into a large gel tray.

ETHIDIUM BROMIDE (10 mg/ml)

Dissolve 0.1 g of ethidium bromide powder in 1 ml of water.

DNA LOADING BUFFER (6 x)

0.25 % bromophenol blue

0.25 % xylene cyanol

30 % glycerol

10 x TRIS ACETATE EDTA (TAE) BUFFER (1L)

48.4 g tris base

20 ml EDTA (0.5 M)

11.42 ml glacial acetic acid

Make up to 1 L with water and autoclave.

10 x TRIS BORATE EDTA (TBE) BUFFER (1 L)

107,8 g tris base

55 g boric acid

7.44 g di-sodium EDTA

Make up to 800 ml with water and pH to 8.3 with boric acid. Make up to 1 L and autoclave.

RUNNING BUFFER (0.5 x TAE)

20 ml 10 x TAE

380 ml water

λ*Bst*E II MARKER

Digest 260 μl λ DNA (0.25 μg/μl) with 24 μl of 10 x buffer D and 10 μl of *Bst*E II enzyme for 3 hours at 60°C. Check that the digest is successful by running 8 μl on a 1 % TAE gel. To 200 μl of the digest, add 550 μl TE buffer (10 mM, pH 8) and 150 μl loading buffer (6 x). Dispense into eppendorfs (300 μl volumes).

λ*Hind* III MARKER

Same as above, but digest λ DNA with *Hind* III enzyme and buffer B (10 x) at 37°C for 3 hours.

2) The construction of the plasmid library

LURIA BROTH (1 L)

10 g tryptone

10 g sodium chloride

5 g yeast extract

Add 800 ml of water. Adjust pH to 7 with sodium hydroxide (5 M) and then make up to 1 L. Autoclave.

LURIA BROTH / AMP PLATES

Same recipe as above, plus 15 g of nutrient agar. pH to 7 with sodium hydroxide (5 M). Autoclave. Add 1 μl of ampicillin (10 mg/ml) per 1 ml of broth.

AMPICILLIN STOCK (100 mg/ml)

Dissolve 1 g of ampicillin powder in 10 ml ddd water. Aliquot into 10 eppendorfs and store at - 20° C.

5 M SODIUM HYDROXIDE (100 ml)

4.20 g sodium hydroxide

100 ml water

IPTG (ISOPROPYLTHIO-β-D-GALACTOSIDE)

0.12 g IPTG

5 ml water

Filter sterilise and dispense 500 μl into eppendorfs. Store at – 20° C.

X-GAL (5-BROMO-4-CHLORO-3-INDOLYL-β-D-GALACTOSIDE)

0.01 g 5-bromo-4-chloro-3-indoyl-β-D-galactoside

2 ml N-N-dimethyl-formamide

Dispense into eppendorfs (500 μl volumes) and cover eppendorfs in foil. Store at – 20°C.

GLYCEROL/LB MEDIA (500 ml)

250 ml glycerol

250 ml LB media

3) Alkaline lysis method for plasmid DNA isolation

White colonies are subcultured in 5 ml LB/AMP test tubes overnight at 37°C. Streak out colonies onto LB/AMP plates, incubated overnight at 37°C.

Plasmid extractions are carried out as follows:

- Transfer 1.5 ml of culture to an eppendorf and centrifuge for 1 minute at 13 000 g. Remove medium by vaccum aspiration, leaving the pellet as dry as possible.
- Resuspend the pellet by vortexing in 100 μl of ice-cold lysozyme solution and stand for 5 minutes at room temperature.
- Add 200 μl of a fresh NaOH/SDS solution and mix by inverting the tube 2-3 times (do not vortex). Store on ice for 5 minutes.
- Add 150 μl of acetate neutralising solution and vortex the tube gently at low speed in an inverted position for 10 seconds. Store on ice for 5 minutes.

- Centrifuge for 5 minutes.
- Remove the supernatant into a clean tube and extract with equal volumes (400 μl) of phenol and chloroform:isoamyl alcohol (24:1). Centrifuge for 5 minutes and remove the upper phase. Re-extract with an equal volume of chloroform:isoamyl alcohol only, centrifuge again and keep the supernatant.
- Add two volumes (900 μl) of 95 % ethanol, mix by vortexing briefly and stand at room temperature for 2 minutes. Centrifuge for 5 minutes at room temperature to obtain a DNA pellet.
- Remove the supernatant, gently rinse the pellet with 500 μl of 70 % ethanol, centrifuge again and air dry the pellet for 15 minutes.
- Add 20 μl of TE/RNase and vortex to dissolve. Incubate at 37°C for 30 minutes. The yield of plasmid is usually 5-10 μg. Store at –20°C.

ACETATE NEUTRALISING SOLUTION (100 ml)

24.9 g potassium acetate

11.5 ml glacial acetic acid

Make up to 100 ml and filter sterilise

LYSOSYME SOLUTION

To 1 ml of the lysosyme solution , add 0.004 g lysosyme.

SODIUM HYDROXIDE /SDS LYSIS SOLUTION (8 ml)

7.28 ml water

0.8 ml 10 % SDS

0.32 ml sodium hydroxide

Make up just before use.

TE/RNASE (1 ml)

1 ml of TE buffer (10 mM, pH 8)

2 μl of RNase A (10 mg/ml).

SMART PREPS

- Add 1.5 ml of culture to an eppendorf.
- Microfuge for 60 seconds.
- Vacuum suck the supernatant till the pellet is dry. Conduct process twice.
- Resuspend pellet in 50 μl Smart buffer.
- Incubate at 37°C for 15 minutes.
- Cool on ice for 5 minutes.
- Boil at 100°C for 90 seconds.
- Microfuge for 10 minutes.
- Transfer supernatant to clean eppendorfs.

SMART BUFFER

100 μl 1 M Tris-HCl, pH 8.0

100 μl 100 mM EDTA

3.75 ml 40 % sucrose

200 μl 5 mg/ml BSA

500 μl 4 mg/ml RNase A

20 mg Lysosyme

Make up to 10 ml with distilled water and dispense into 0.5 ml aliquots. Freeze at – 20° C.

4) Transformations

PREPARATION OF COMPETENT *E.coli* CELLS (HEAT SHOCK)

Competent *E.coli* cells were prepared by inoculating 5 ml of LB with DH5α cells. These are allowed to grow overnight at 37°C. A 100-μl aliquot was plated onto an LB agar plate. The cells, which had grown to confluence the following morning, were scraped off the agar plate and added to LB broth. Four LB flasks of 100 ml each were inoculated with 1.5, 1, 0.7 and 0.3 ml of the pre-inoculum and incubated at 37°C on the orbital shaker for approximately 2 hours. The cultures were allowed to grow till an absorbance of 0.6–0.8 (OD_{600}). The flasks were then cooled for 5-10 minutes in an ice waterbath. The cultures were then centrifuged using rotor JA 14 and spun at 5 000 rpm, for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 50 ml of RF1 (100 mM KCl, 50 mM $MnCl_2$, 30 mM CH_3COOK, 10 mM $CaCl_2$, 15 %

m/v glycerol, pH 5.8). This was then incubated on ice for 20 minutes. The cells were pelleted by a 10 minute centrifugation as above. The supernatant was decanted and the four pellets were resuspended in 4 ml RF2 (10 mM Mops, 10 mM KCl, 75 mM $CaCl_2$, 15 % m/v glycerol, pH 6.8) and the flask contents were pooled. Aliquots in multiples of 150 μl were pipetted into sterile Eppendorf tubes and were stored at – 80°C, until use.

METHOD

- Add 150 μl of thawed competent *E.coli* to DNA ligation mix (2.5 μl). Mix.
- Leave on ice for 20 minutes.
- Heat-shock at 42°C for 45 seconds and then cool on ice for 5 minutes.
- Add 1 ml of SOC medium and incubate at 37°C for 1 hour.
- Plate all cells on LB/AMP plates (4 μl IPTG and 40 μl X-gal).
- Incubate plates overnight at 37° C.

PREPARATION OF ELECTROCOMPETENT *E.coli* CELLS (ELECTROPORATION)

- Set up a 50 ml culture of *E.coli* cells (DH5α) from a fresh LB plate and incubate overnight with shaking at 37°C.
- Inoculate a 500 ml culture with 50 ml O/N culture. Grow for several hours with shaking until the $OD_{600} = 0.65$.
- Place on ice for 30 minutes (cells must remain on ice for the remainder of the procedure).
- Collect cells by centrifuging at 6 750 rpm for 15 minutes at 4°C.
- Resuspend the cells in 500 ml of ice-cold sterile Millipore water.
- Collect cells by centrifuging at 6 750 rpm at 4°C for 15 minutes.
- Resuspend cells in 250 ml of ice-cold sterile Millipore water.
- Collect cells by centrifuging for 15 minutes at 6 750 rpm at 4°C.
- Resuspend cells in 10 ml of 10 % glycerol, which has been diluted using sterile Millipore water. Collect cells by centrifuging at 6 750 rpm for 15 minutes at 4°C.
- Resuspend the cells in 1.5 ml of 10 % glycerol, which has been diluted with sterile Millipore water.

• Aliquot 40 μl quantities into sterile Eppendorfs and freeze immediately using liquid nitrogen. Store at – 80°C.

METHOD

• Add 50 μl of ddd water to 10 μl of the ligation reaction and incubate at 68°C for 1 minute.

• Mix the ligation and allow to cool.

• Remove electrocompetent cells from the – 80°C freezer and add 80 μl ddd water.

• Set the electroporator to 1800 V.

• Add 40 μl of electrocompetent cells to 1.5 μl of ligation reaction and mix.

• Add to the cuvette. Tap the cuvette such that the mix sinks to the bottom of the tube.

• Wipe the outside of the cuvette.

• Place the cuvette into the electroporation arm and place into the electroporator.

• Press the pulse button twice. Once the machine buzzes, remove the cuvette and add 200 μl of ice-cold SOC medium. Mix and return to the original eppendorf.

• Place tubes at 37°C for 30 minutes (to allow cells to recover).

• Plate 200 μl of the transformation onto LB/AMP plates that have 4 μl of IPTG and 40 μl of X-gal.

SOC MEDIUM

1.0 g tryptone

0.5 g yeast extract

0.05 g sodium chloride

10 ml potassium chloride (250 mM)

Dissolve in 85 ml of distilled water, pH to 7.0 and adjust the volume to 98 ml. Sterilise by autoclaving. Once the solution is cool, add 1 ml magnesium chloride (2 M) and 1 ml glucose stock (2 M) that has been filter sterilised. The final concentration must be 20 mM. Filter the complete medium through a 0.2 mm filter unit.

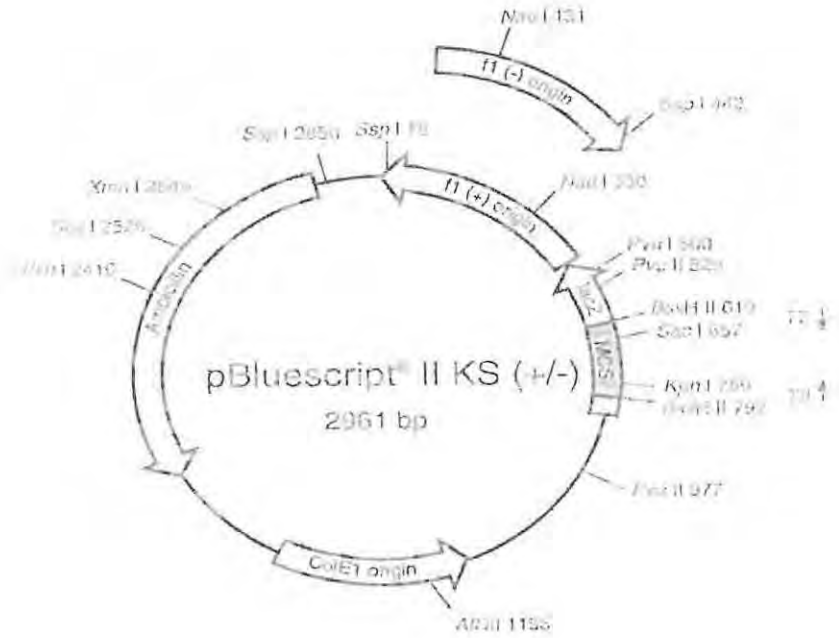


Figure 50: pBluescript (SK+) plasmid vector (Stratagene) used for cloning of *Eco*R1 and *Bam*H1 fragments for the construction of the plasmid library.

APPENDIX 3: PHYSICAL MAPS OF THE CIGV GENOME

1) DIG solutions

20 x SSC (1L)

175.3 g sodium chloride

88.2 g sodium acetate

Make the solution up to 800 ml with water and pH to 7. Make up to 1 L and autoclave.

MALEIC ACID BUFFER (1L)

11.607 g maleic acid

8.766 g sodium chloride

Make up the solution to 800 ml with water and pH to 7 with solid sodium hydroxide. Make up to 1 L and autoclave.

WASHING BUFFER (1L)

3 ml of Tween-20

997 ml maleic acid buffer.

DETECTION BUFFER (1L)

12.11 g tris base

5.844 g sodium chloride

Make up to 800 ml with water and pH to 9.5. Make up to 1 L and autoclave.

STRIPPING SOLUTION (500 ml)

8.401 g sodium hydroxide

0.5 g SDS

Make up to 500 ml with water and autoclave.

BLOCKING SOLUTION (100 ml)

To 10 ml of the blocking solution stock (DIG High Prime DNA Labelling and Detection Starter Kit II - Roche), add 90 ml of maleic acid buffer.

ANTI-DIG SOLUTION (20 ml)

Add 2 μl of anti-DIG (vial 4 - DIG kit) to 20 ml of blocking solution.

2 x SSC, 0.1 % SDS (500 ml)

50 ml 20 x SSC stock

450 ml water

0.5 ml 0.1 % SDS

0.5 x SSC, 0.1 % SDS (500 ml)

12.5 ml 20 x SSC stock

487.5 ml water

0.5 ml 0.1 % SDS

DIG EASY HYBRIDISATION BUFFER

To make up the hybridisation buffer, add 64 ml ddd water to DIG Easy Hyb Granules. Allow the granules to dissolve by stirring on a heating block at 37°C for 10 minutes.

ddd Water (50 ml)

50 ml of dd water is autoclaved.

2) Southern blotting solutions

DEPURINATION SOLUTION (1 L)

250 ml HCl (0.25 M)

750 ml water

DENATURATION SOLUTION (1 L)

87.66 g sodium chloride (1.5 M)

20 g sodium hydroxide (0.5 M)

NEUTRALISATION SOLUTION (1 L)

87.66 g sodium chloride (1.5 M)

60.58 g tris base (0.5 M)

0.372 g EDTA (0.001 M)

pH to 7.5 with HCl.

3) Nucleospin extraction 2 in 1 (Macherey – Nagel)

Procedure for DNA extraction from TAE agarose gels:

300 μl of buffer NT1 is added for each 100 mg of agarose gel in an eppendorf. The sample is incubated at 50°C for 10 minutes, vortexing briefly every 2–3 minutes. Next the sample is loaded onto a nucleospin column, which is placed in a 2 ml collection tube and centrifuged for 60 seconds at 10 000 rpm.

The flow through is discarded and the spin column placed into the collection tube. 600 μl of buffer NT3 is added and the column is centrifuged for 1 minute at maximum speed. This washing step is repeated.

The flow through is discarded and the column placed into the collection tube. 200 μl of buffer NT3 is added and the column is centrifuged at maximum speed for 2 minutes to remove residual alcohol.

Finally 50 μl of Elution buffer (NE) is added and allowed to incubate for 1 minute. The column is then spun at maximum speed for 1 minute to elute the DNA.

4) Physical maps

Table 17: *Bam*H1 restriction fragments of ClGV DNA and their secondary *Xho*1 fragments.

	1°Enzyme: single	**Digest *Bam*H1**	**2°Enzyme: *Xho*1**	**Double digest**
FRAGMENT	FRAGMENT	SIZE (kbp)	FRAGMENTS	SUMMATION OF FRAGMENT SIZES (kbp)
1	A	32.0	b, j, f, i	32.0
2	B	24.9	a, k	24.9
3	C	11.5	c	11.5
4	D	9.85	d	9.85
5	E	9.70	e	9.70
6	F	7.90	g	7.90
7	G	7.50	h	7.50
8	H	6.10	m, l	6.20
9	I	2.60	n	2.60
		112.13		112.15

Table 18: *Xho1* restriction fragments of ClGV DNA and their secondary *Bam*H1 fragments.

	1°Enzyme: single	**Digest *Xho*1**	**2°Enzyme: *Bam*H1**	**Double digest**
FRAGMENT	FRAGMENT	SIZE (kbp)	FRAGMENTS	SUMMATION OF FRAGMENT SIZES (kbp)
1	A	57.5	c,d,e,g,h,m,n,l,k	58.63
2	B	23.5	a	23.5
3	C	14.5	f,i	14.05
4	D	12.1	b	12.10
5	E	4.65	j	4.65
		112.25		112.93

Table 19: *Bam*H1 restriction fragments of ClGV DNA and their secondary *Sac*1 fragments.

	1°Enzyme: single	**digest *Bam*H1**	**2°Enzyme: *Sac*1**	**Double digest**
FRAGMENT	FRAGMENT	SIZE (kbp)	FRAGMENTS	SUMMATION OF FRAGMENT SIZES (kbp)
1	A	32.0	a,c	34.2
2	B	24.9	b,i,j	23.85
3	C	11.5	d	11.5
4	D	9.85	e	9.85
5	E	9.70	f	9.70
6	F	7.90	g	7.90
7	G	7.50	h	7.50
8	H	6.10	k.l	6.20
9	I	2.60	m	2.60
		112.13		113.3

Table 20: *Sac*1 restriction fragments of ClGV DNA and their secondary *Bam*H1 fragments.

	1°Enzyme: single	**digest *Sac*1**	**2°Enzyme: *Bam*H1**	**Double digest**
FRAGMENT	FRAGMENT	SIZE (kbp)	FRAGMENTS	SUMMATION OF FRAGMENT SIZES (kbp)
1	A	33.0	b,c,i	32.05
2	B	30.4	d,f,h,l	31.7
3	C	27.5	e,g,k,m,j	27.55
4	D	22.0	a	22.0
		112.90		113.3

Table 21: *Xho1* restriction fragments of ClGV DNA and their secondary *Sac*1 fragments.

	1°Enzyme: single	**digest *Xho*1**	**2°Enzyme: *Sac*1**	**Double digest**
FRAGMENT	FRAGMENT	SIZE (kbp)	FRAGMENTS	SUMMATION OF FRAGMENT SIZES (kbp)
1	A	57.5	a,b,g	56.16
2	B	23.5	c,e	25.32
3	C	14.5	f,i,j	14.65
4	D	12.1	d	12.1
5	E	4.65	h	4.65
		112.25		112.88

Table 22: *Sac*1 restriction fragments of ClGV DNA and their secondary *Xho*1 fragments.

	1°Enzyme: single	**digest *Sac*1**	**2°Enzyme: *Xho*1**	**Double digest**
FRAGMENT	FRAGMENT	SIZE (kbp)	FRAGMENTS	SUMMATION OF FRAGMENT SIZES (kbp)
1	A	33.0	c,e,j,i	32.79
2	B	30.4	a	30.4
3	C	27.5	g,b	25.76
4	D	22.0	d,f,h	24.88
		112.90		113.83

Table 23: *Kpn*1 restriction fragments of ClGV DNA and their secondary *Sac*1 fragments.

	1°Enzyme: single	**digest *Kpn*1**	**2°Enzyme: *Sac*1**	**Double digest**
FRAGMENT	FRAGMENT	SIZE (kbp)	FRAGMENTS	SUMMATION OF FRAGMENT SIZES (kbp)
1	A	27.0	a,j	25.0
2	B	22.95	g,h,f	24.74
3	C	16.20	b	16.20
4	D	15.00	c	15.00
5	E	11.83	o	11.83
6	F	10.0	e	10.0
7	G	3.3	i	3.3
8	H	2.3	k	2.3
9	I	1.9	l	1.9
10	J	1.65	m	1.65
11	K	1.10	n,o	1.62
		112.48		113.54

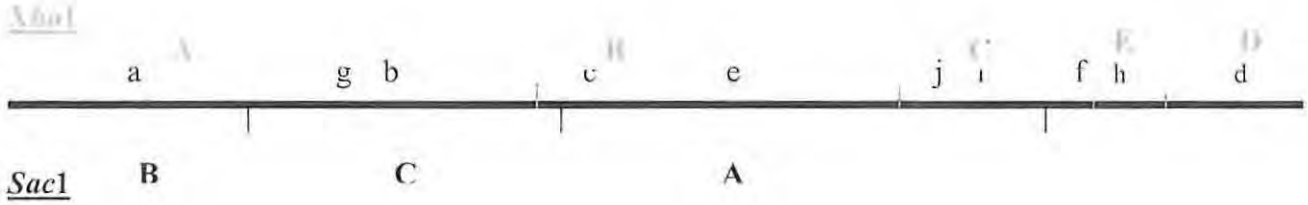
Table 24: *Sac*1 restriction fragments of ClGV DNA and their secondary *Kpn*1 fragments.

	1°Enzyme: single	**digest *Sac*1**	**2°Enzyme: *Kpn*1**	**Double digest**
FRAGMENT	FRAGMENT	SIZE (kbp)	FRAGMENTS	SUMMATION OF FRAGMENT SIZES (kbp)
1	A	33.0	c,e,f,i	37.45
2	B	30.4	d,b,l,m	32.95
3	C	27.5	g,h,j,k,n,o,i	22.19
4	D	22.0	a	22.0
		112.90		114.59

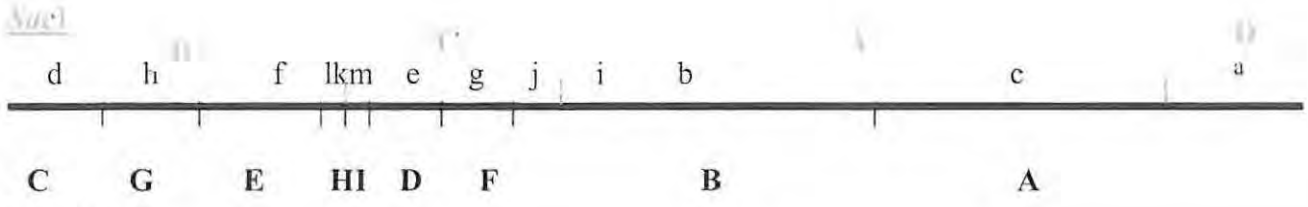
Figure 51: Restriction maps of ClGV-SA. The upper case letters denote single digest fragments, which are above and below the maps and the lower case letters denote the double digest fragments of the two enzymes. Maps are not drawn to scale.

A = *Xho*1 and *Sac1* B = *Bam*H1 and *Sac*1

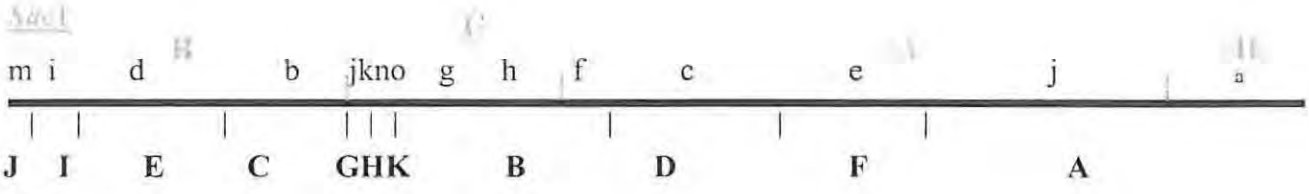
C = *Sac*1 and *Kpn*1 D = *Xho*1 and *Bam*H1



(A)

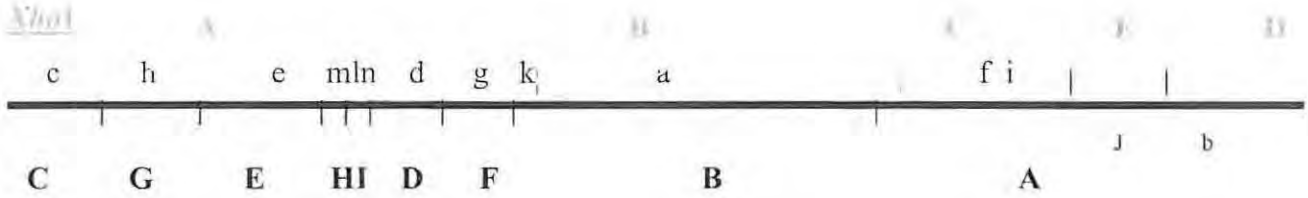


(B)



*Kpn*1

(C)



*Bam*H1

(D)

APPENDIX 4: THE IDENTIFICATION AND CHARACTERISATION OF THE GRANULIN AND *egt* GENES

1) PCR

10 mM dNTP'S

30 µl of each nucleotide (dATP, dTTP, dCTP & dGTP) is added into an eppendorf. 180 µl of ddd water is added, bringing the total volume to 300 µl. Aliquot into eppendorfs (60 µl volumes) and store at -20°C.

GENERAL MIXES

Two master mixes are prepared as follows:

Master Mix 1:

2.0 µl 10 mM dNTP's

x µl Template DNA (0.1 - 0.75 g)

1.5 µl Forward Primer

1.5 µl Reverse Primer

x µl ddd water

25 µl

Master Mix 2:

5 µl Expand Buffer (10 x)

0.75 µl Expand enzyme (Roche)

19.25 µl ddd water

25 µl

Mix the two mixes together and place in the thermocycler.

PCR THERMOCYCLER SETTINGS – PCR*Sprint* (Hybaid)

1 minute at 95°C

30 seconds at 95°C ┐

2 minutes at 52°C │

1 minute at 72°C │ Repeat cycle 30 x

1 minute at 72°C │

1 minute at 25°C ┘

Hold temperature – 4°C

2) Sequencing reactions

QIAPREP PLASMID PURIFICATION KIT (QIAGEN)

♦ Pellet bacterial cells from overnight cultures by centrifuging for 2 minutes at 13 000 rpm. Resuspend cells in 250 µl of buffer P1.

♦ Add 250 µl of buffer P2 and gently invert the tube 4-6 times to mix.

♦ Add 350 µl of buffer N3 and invert the tube immediately but gently 4-6 times.

♦ Centrifuge for 10 minutes.

♦ Apply the supernatant to a QIAprep spin column in a 2 ml collection tube.

♦ Centrifuge for 60 seconds and discard the flow-through.

♦ Wash the spin columns by adding 0.75 ml of buffer PE and centrifuge for 60 seconds.

♦ Discard the flow-through and centrifuge for an additional 1 minute to remove residual wash buffer.

♦ Place the QIAprep column in a clean microfuge tube and add 50 µl of 10 mM Tris-HCl (pH 8.5) or water, to the centre of the column and stand for 1 minute.

♦ Centrifuge for 1 minute to elute the DNA.

THERMOCYCLING USING THE BIG DYE TERMINATOR CYCLE SEQUENCING KIT (Applied Biosystems)

Make up the volume of the PCR product/Clone to 12 μl. DNA concentration must be between 5-20 ng of PCR product and 200-500 ng of plasmid. Add primer (0.6 μl of 10 μM primer) and distilled water (to volume of 12 μl). Add 8 μl of BIG DYE. Place in thermocycler (GeneAmp 9700) and cycle DNA as follows:

- Rapid thermal ramp to 96°C.
- 96°C for 10 seconds.
- Rapid thermal ramp to 50°C
- 50°C for 5 seconds.
- Rapid thermal ramp to 60°C.
- 60°C for 4 minutes.
- Cycle is repeated 25 times.
- Rapid thermal to 4°C and hold.

DNA CLEAN UP AND CONCENTRATION USING ZYMO COLUMNS (ZYMOGEN)

♦ Add 2 volumes of DNA binding buffer to sample.

♦ Load onto a zymo column and place in a 2 ml collection tube.

♦ Centrifuge at 10 000 g for 10 seconds. Discard the flow-through.

♦ Add 200 μl of Wash buffer to the column and centrifuge for 30 seconds at maximum speed.

♦ Discard the flow-through and the repeat washing step.

♦ Add 8 μl of triple distilled water directly to the column matrix. Place in an eppendorf and centrifuge briefly to elute the DNA.

♦ Place the DNA into the vaccum centrifuge for 1 hour (till the liquid has evaporated).

♦ Store the sample at –20°C till ready for sequencing.

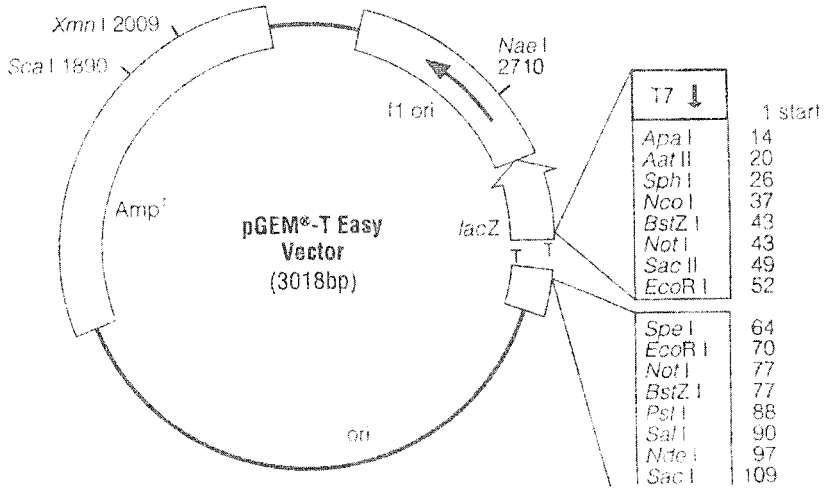


Figure 52: pGEM- T Easy plasmid vector (Promega) used to clone *egt* PCR products.

3. Molecular weight marker

Table 25: Molecular weights of fragments of the 1 kb ladder marker

FRAGMENT	SIZE (kbp)
1	12 216
2	11 198
3	10 180
4	9 162
5	8 144
6	7 126
7	6 108
8	5 090
9	4 072
10	3 054
11	2 036
12	1 636
13	1 018
14	517, 506
15	398, 394
16	298

APPENDIX 5: DETECTION ASSAYS FOR *Cryptophlebia leucotreta* GRANULOVIRUS IN FALSE CODLING MOTH LARVAE

1) Dot blots

10 % SDS (100 ml)

10 g sodium dodecyl sulphate

100 ml water

0.2 M SODIUM HYDROXIDE (100 ml)

0.8 g sodium hydroxide

100 ml water

15 x SSC BUFFER (20 ml)

15 ml 20 x SSC buffer

5 ml water

6 x SSC BUFFER (20 ml)

6 ml 20 x SSC buffer

14 ml water

2) Radioactive labelling

20 mM dNTP-CTP (200 µl)

TTP (100 mM) 40 µl

GTP (100 mM) 40 µl

ATP (100 mM) 40 µl

ddd water 80 µl

DNA POLYMERASE 1

8 µl ddd water

1 µl 10 x nick translation buffer

1 µl DNA polymerase 1 (10 units/µl)

MEDIUM STRINGENCY WASHES

2 x SSC, 0.1 % SDS (500 ml)

50 ml 20 x SSC

450 ml ddd water

0.5 g SDS

Heat to 42°C in order to dissolve the SDS.

4 x SSC, 0.1 % SDS (400 ml)

80 ml 20 x SSC

320 ml ddd water

0.4 g SDS

Heat to 42°C in order to dissolve the SDS.

0.5 x SSC, 0.1 % SDS (400 ml)

10 ml 20 x SSC

390 ml ddd water

0.4 g SDS

Heat to 42°C in order to dissolve the SDS.

10 x NICK TRANSLATION BUFFER (10 ml)

10 ml 0.5 M Tris-HCl (pH 7.5)

0.12 g 0.1 M Mg_2SO_4

0.0015 g 1 mM dithiothreitol

500 µg/ml BSA

Divide into smaller aliquots of 1 ml and store at – 20°C.

0.5 M EDTA (pH 8.0) (10 ml)

1.86 g EDTA

10 ml ddd water

20 x SSC STOCK SOLUTION (1 L)

175.3 g sodium chloride

88.2 g sodium citrate

Add 800 ml of water and adjust the pH to 7 with sodium hydroxide (5 M). Make up to 1 L and sterilise by autoclaving.

PREHYBRIDISATION / HYBRIDISATION BUFFER (30 ml)

0.1266 g KH_2PO_4

0.075 g SDS (0.25 % of volume)

1.5 g dextran sulphate (5 % of the volume)

15 ml deionised formamide

1.5 ml Denhardts solution (50 x)

20 x SSC (5 x final volume)

6.9 ml DEPC water

50 x DENHARDTS SOLUTION (50 ml)

0.5 g ficoll

0.5 g polyvinylpyrrolidone

0.5 g BSA

Make up volume to 50 ml with ddd water and store at – 20°C.

DEIONIZED FORMAMIDE

Add 20 g amberlite resin for 200 ml of formamide (1/10th the volume). Stir for 1 hour and filter through Whatmans no. 1 twice and store at 4°C.

5 % TCA/SODIUM PYROPHOSPHATE

2.5 g trichloro-acetic acid

3.3 g sodium pyrophosphate

0.5 M EDTA (pH 8)

18.337 g EDTA

100 ml water

pH to 8.0

QUICK SPIN COULMNS, SEPHADEX G-50 (Roche)

- Gently invert the column several times to resuspend the medium.

- Remove the upper and lower caps of the column and allow the buffer to drain by gravity.
- Place the column in a collection tube and centrifuge for 2 minutes at 1100 x g in a swinging bucket rotor. The eluted buffer is discarded.
- Apply the radiolabelled probe to the centre of the column, keeping the column upright.
- Place the column in a collection tube and centrifuge for 4 minutes at 1100 x g.
- The elutant contains the purified probe and is stored in a lead container at –20°C. The column is discarded into radioactive waste.

3) PCR solutions

HOMOGENISATION BUFFER (100 ml)

5 g ascorbic acid

10 g sodium dodecyl sulphate (SDS)

0.6 g Tris-HCl, pH 7.7 (10 mM)

0.186 g EDTA (1 mM)

ALKALI SOLUTION (500 ml)

42.92 g sodium carbonate (0.3 M)

2.23 g EDTA pH 8 (0.03 M)

14.66 g sodium chloride (0.51 M)

1 M TRIS-HCl (pH 8) (500 ml)

60.55 g tris base

500 ml water.

pH to 8 with HCl. Sterilise by autoclaving.

REFERENCES

Akiyoshi, D, Chakerian, R & Rohrmann, M.H. (1985). Cloning and Sequences of the Granulin Gene from *Trichoplusia ni* Granulosis Virus. Virology, **141**, 328-332.

Annecke, P & Moran, C (1982). Insects and mites of cultivated plants in South Africa. Butterworth Publishing.

Barrett, J.W, Krell, P.J & Basil, M.A (1995). Characterisation, sequence 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.

Blissard, T & Rohrmann, M (1990). Baculoviruses diversity and molecular biology. Annual Review Entomology, **35**, 127-135.

Brown, S.R, Marunaik, J.E & Kudson, D.L (1993). Baculoviruses (MNPV) and their genomic variants. Journal of General Virology, **66**, 2431-2433.

Carstens, E.B (1996). http://www.queensu/micr/micr221/Insectviruses.html.

Chen, X, Sun, X, Hu, Z, Li, M, O'Reilly, D.R, Zuidema, D & Vlak, J.M (2000). Genetic Engineering of *Helicoverpa armigera* Single Nucleocapsid Nuclearpolyhedrovirus as an Improved Pesticide. Journal of Invertebrate Pathology, **76**, 140-146.

Clarke, S.E, Tristern, M, Cory, J.S & O'Rielly, D.R (1996). Characterisation of the ecdysteroid UDP-glucosyltransferase gene from *Mamestra brassicae* nucleopolyhedroviruses. Journal of General Virology, **77**, 2856-2871.

Consigli, R.A, Russel, D.K & Wilson, M.C (1986). Biochemistry and Molecular Biology of Baculoviruses that infect *Plodia interpunctella*. Molecular biology of Baculoviruses, 69-92, Berlin & Springer.

Crook, N.E (1981). A comparison of Granulosis Viruses from *Pieris brassicae* and *Pieris rapae*. Virology, **115**, 173-181.

Crook, N.E (1985). Variation in *Cydia pomenella* GV isolates and physical maps of DNA from three variants. Viruses of Invertebrates, **55**, 73 – 110.

Crook, N.E. (1986). Restriction Enzyme Analysis of Granulosis Viruses isolated from *Arjogeia rapae* and *Pieris brassicae*. Journal of General Virology, **66**, 2430-2432.

Crook, N.E (1991). Baculoviridae: subgroup B. comparative aspects of granulosis viruses. Encyclopaedia of Virology, Volume 1, Edited by R.E Webster & A. Granoff. Academic Press.

Crook, N.E, Spencer, R.A, Payne, C.C & Lewy, D.J (1985). Variation in *Cydia pomenella* Granulosis virus Isolates and Physical Maps of the DNA from Three variants. Journal of Virology, **66**, 2423-2430.

Crook, N.E, Chem, R.J & Miller, L.K (1993). An Apoptosis Inhibiting Baculovirus Genes with Zinc Finger-Like Motif. Journal of Virology, **67**, 2186-2174.

Crook, N.E, James, J.D, Smith, I.R.L & Winstanely, D (1997). Comprehensive physical map of the *Cydia pomonella* granulovirus genome and sequence analysis of the granulin gene region. Journal of General Virology, **78**, 965-974.

DIG Instruction manual (2000). Roche Biochemicals, Mannheim, Germany.

Doerfler & Bohm (1986). The molecular biology of Baculoviruses. Springer-Verlag .

Dwyer, K.G & Grandos, R.R (1987). A Physical Map of *Peris rapae* Granulosis Virus Genome. Journal of General Virology, **68,** 1471 - 1476.

Eldridge, R, O'Reilly, D.R, Hammock, B.D & Miller, L.K (1992). Insecticidal Properties of Genetically Engineered Baculoviruses Expressing an Insect Juvenile Hormone Esterase Gene. Applied and Environmental Microbiology, **58**, 1583-1591.

Farrell, R.E (1993). RNA methodologies: a laboratory guide for the isolation and characterisation of RNA. Academic Press.

Frankel-Conrat, H, Kimball, P.C & Levy, J.A (1998). Virology (2nd Edition). Prentice Hall Inc.

Flipsen, J.M, Mans, R.M.W, Kleefsmon, A.W.F, Knebel - Morsdorf, D & Vlak. J.M (1995). Deletion of Baculoviral EGT gene induces early disintegration of the malphagion tubules of infected insects. Journal of Virology, **69**. 4529-45.

Gettig, R.R & McCarthy, W.J (1982). Genotypic variation among isolates of *Heliothis* species nuclear polyhedrosis viruses from different geographical areas. Virology, **117**, 245 - 252.

Goto, C, Minobe, Y & Ilzuka, T (1992). Restriction endonuclease analysis and mapping of the genomes of granulosis viruses isolated from *Xestia c-nigrum* and five other noctuid species. Journal of General Virology, **73**, 1491-1497.

Granados, R.R & Williams, K.A. (1986). *In vivo* pathway of *Autographa californica* Baculovirus invasion and infection. Virology, **108**, 297-308.

Harris, R.C.J (1964). Techniques in experimental virology. Academic Press.

Hashimoto, Y, Bartholomew, G.C & Granados, R.R (1991). Location and nucleotide sequence of the gene encoding the viral enhancing factor of the *Trichoplusia ni* granulosis virus. Journal of General Virology, **72**, 2645-2651.

Hashimoto, Y. Hayashi, K, Okuna, Y, Hayakawa, T, Saimoto, A, Granados, R.R & Matsumoto, T (1996). Physical mapping and identification of interspersed homologous sequences in the *Trichoplusia ni* granulosis virus genome. Journal of General Virology, **77**, 555-563.

Hashimoto, Y, Hayakawa, T, Ueno, Y, Fujita, T, Sano, Y & Matsumoto, T (2000). Sequence Analysis of the *Plutella xylostella* Granulovirus genome. Virology, **275**, 358-372.

Ignoffo, C.M & Heimpel, A.M (1965). The NPV of *Iteliontnis zea* and *Heliothis viresens* - pathogenicity of virus to white mice and guinea pigs. Journal of Invertebrate Pathology, **70,** 329-346.

Jehle, J & Fritsch, E (1992). Physical maps of *Cryphtophlebia leucotreta* Granulosis Virus genome and its relation to the genome of *Cydia pomenalla* Granulosis virus. Journal of General Virology, **136**, 1621-1626.

Jehle, J.A & Backhaus, H (1994). The granulin gene region of the *Cryptophlebia leucotreta* granulosis virus: sequence analysis and phylogenetic considerations. Journal of General Virology, **75**, 3667-3671.

Keating, S.T, Bumand, J.P & Elkington, J.S (1989). DNA Hybridization Assays for the Detection of Gypsy Moth Nuclear Polyhedrosis Viruses in Infected Gypsy Moth (*Lymantria dispar*) Larvae. Applied and Environmental Microbiology, **58**, 2479 - 2484.

Kelly, D.C, Edwards, M.L, Evans, H.F & Robertson, J.S (1978). The Use of the Enzyme Linked Immunosorbent Assay to Detect a Nuclear Polyhedrosis Virus in *Heliothis armigera* Larvae. Journal of General Virology, **40**, 465- 469.

Maeda, S, Volrath, S.L, Hanzlik, T.N, Harper, A, Majima, K, Maddox, D.W, Hammock, B.D & Fowler, E (1991). Insecticidal Effects of an Insect-Specific Neurotoxin Expressed by a Recombinant Baculovirus. Virology, **184**, 777-780.

Maeda, S & Majima, K (1990). Molecular cloning and physical mapping of the genome of *Bombyx mori* nuclear polyhedrosis virus. Journal of General Virology, **71**, 1851-1853.

Mahy, B.W.J & Kungro, H.O (1996). Virology methods manual. Academic Press.

Majima, K, Kobarav, R & Maeda, S (1993). Divergance and evolution of homologous regions of *Bombayx mori* NPV. Journal of Virology, **67**, 7513 – 9518.

Marunik, J.E, Brown, S.C & Kudson, D.V (1984). Physical maps of SFMNPV Baculovirus DNA and its Genomic variants. Virology, **136**, 221-234.

Maramorosch, K (1977). Atlas of insect and plant viruses. Academic press.

Maramorosch, K & Koprowski, H (1967). Methods in virology (Vol 4). Academic Press.

Mikhailov, V.A, Marlyerka, A, Ataeva, J.O, Kullyev, P.K, & Atrazrev, A.M (1986). Characterisation of 3' to 5' exonucleases associated with the DNA Polymerase of Silkworm NPV. Nucleic Acids Research, **14**, 3841- 3850.

Miller, L.K, Fraenkel-Conrat, H & Wagner, R.R (1997). The Baculoviruses. Plenum Press.

Miller, L.K & Dawes, F (1978a). Restriction enzyme analysis for the identification of Baculoviral insecticides. Applied and Environmental Microbiology, **35**, 411 – 421.

Moraes, R.R, Funderburk, J.E & Maruniak, J.E (1998). Polymerase Chain Reaction Technique to Detect Multiple Nuclearpolyhedroviruses in *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) and Predator Populations in Soybeans. Environmental Ecology, **27**, no.4, 968-975.

Moore, S (2000). Personal communication.

Newton, P.J (1998). Lepidoptera: Butterflies & Moths - Citrus pests in the Republic of South Africa. Institute for Tropical and Subtropical Crops.

O'Reilly, D.R & Miller, L (1989). A Baculovirus Blocks Insect Moulting by Producing Ecdysteroid UDP-Glycosyl Transferase. Science, **245**, 1110 - 1118.

O'Reilly, D.R & Miller, L.K (1990). Regulation of Expression of a Baculovirus Ecdysteroid UDP-Glucosyltransferase Gene. Journal of Virology, **64**, 1321-1328.

O'Reilly, D.R, Miller, K, Luckow, V.A & Freeman, W.H (1992). Baculovirus expression vectors. Prentice Hall Inc.

O'Reilly, D.R & Miller, L (1992). Improvement of a Baculoviral pesticide by deletion of the EGT gene. Bio/Technology, **9**, 1086-1089.

Possee, R.D & Kelly, D.C (1988). Physical Maps and Comparative DNA Hybridisation of *Mamestra brassicae* and *Panolis flammea* Nuclear Polyhedrosis Virus Genomes. Journal of General Virology, **69**, 1285-1298.

pGEM T and pGEM T Easy Vector systems (1998). Promega, Madison, WI, USA.

Pinhey, E.C.G (1975). Moths of Southern Africa. Tafelberg Publishers.

Qiagen Tip Handbook (1997). Qiagen, Hilden, Germany.

Riegel, C.I, Lanner-Herrera, C & Slavicek, J.M (1994). Identification and characterisation of the ecdysteroid UDP-glucosyltransferase gene of the *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus. Journal of General Virology, **75**, 829-838.

Rotel, D.J & Faulkner, P (1984). The course analysis and mapping of *Autographa Californica* Nuclear Polyhedrosis Virus transcripts. Journal of Virology, **50**, 739 – 747.

Sambrook, J, Fritsch, E.F & Maniatis, T (1989). Molecular cloning- a laboratory manual (2nd Edition). Cold Spring Harbour Laboratory Press.

Smith, K.M (1967). Insect Virology . Academic Press.

Smith, R.E (1971). Viruses causing the polyhedrosis and granulosis viruses in insects. Comparative Virology. Academic Press.

Smith, R.E & Crook, N.E (1988). Physical Maps of Genomes of four Variants of *Artogeria rapae* Granulosis virus. Journal of General Virology, **69**, 1741-1747.

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

Spillings, B (2000). BSc Honours Thesis: An investigative analysis of the *Cryptophlebia leucotreta* Granulosis Virus of the *Cryptophlebia leucotreta*, False Codling Moth. Rhodes University.

Stewart, L.M.D, Hirst, M, Lopez-Ferber, M, Merrywaether, A.T, Cayley, P.J & Possee, R.D (1991). Construction of an improved baculovirus insecticide containing an insect specific toxin gene. Nature, **352**, 83-85.

Summers, M, Engeler, R, Falcon, L.A & Vail, P (1975). Baculoviruses for pest control : Safety considerations. American Society for Microbiology.

Tanada Y & Kaya H, (1993). Insect Pathology. Academic Press.

Tjia, S.T, Corstens, E.C. & Doerfler, W (1979). Infection of *Spodoptera Frugperdai* cells with *Autographa Californica* NPV II. - The Viral DNA and the kinetics of its replication, Virology. **99**, 391 – 409

Tracey, M.D & All, J.N (1996). Impact on Insect species- Aahit gene insertion on inherent bioactivity of Baculoviruses against tobacco budworm *Heliothis vinescent* and cabbage looper *Trichoplusia ni.* Proceedings of the Beitwide cotton Production and research conference . 911-917

Tweeten, K.A, Bulla, L.A & Consigli, R.A (1980). Restriction Enzyme Analysis of the Genomes of *Plodia interpunctella* and *Pieris rapae* Granulosis Viruses. Virology, **104**, 514-519.

Unknown 1 (2000). http://www.hortnet.co.nz

Volkman, L.E, Goldsmith, P.A, Hein, R.T & Faulkner, P (1995). Neutralisation of budded *Autographa californica* Nuclear Polyhedrosis virus by a monoclonal antibody of the target antigen. Virology, **133**, 354-362.

Webster, R.A. & Granoff, A (1999). Encyclopaedia of Virology (2nd edition). Academic Press.

Weeden, C (1996). http://www.aruba.nysaes.cornell.edu/ent/biocontrol/pathogens/viruses.html

Wormealton, S.L & Winstanely, D (2001). Phylogenetic analysis of conserved genes within the EGT region of the slow-killing *Adoxophyes orana* granulovirus. Journal of General Virology, 82, 2295-2305.