The characterization of African horsesickness virus VP7 particles with foreign peptides inserted into site 200 of the VP7 protein top domein.

By

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Submitted in partial fulfilment of the requirements for the degree MSc in the Faculty of Natural and Agricultural Science

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Déwald, my friends and the J-Vic group
SUMMARY

The characterization of African horsesickness virus VP7 particles with foreign peptides inserted into site 200 of the VP7 protein top domein.

By

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Promoters: Professor Henk Huismans and Dr Wilma Fick
Department of Genetics
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For the degree MSc

African horsesickness virus (AHSV) VP7 development as a particulate vaccine system, for human and veterinary use, is based on the observation that VP7 forms hexagonal crystalline structures which can be exploited to present epitopes to the immune system. VP7 can be produced in large amounts by means of the baculovirus expression system and is easily purified. In order to display an epitope or small peptides in the VP7 top domain several VP7 display vectors have been constructed that will allow the insertion of peptides into at least three different amino acid sites. These vectors are VP7mu144, VP7mu177 and VP7mu200. Vector VP7mu177 in which different peptides were inserted into site 177 has been investigated by Rutkowska (2002). The aim of this investigation is to insert the same peptides into site 200 and compare the constructs with regard to assembly into VP7 particles, structure, stability and antigenicity. These VP7 vectors have to be characterized to determine which of these mutant sites will elicit the best immune response.

Insertion mutants were made of the major core protein of AHSV VP7 serotype 9 at specific amino acid positions for the possible use as a subunit vaccine by F. Maree (2000). In this study HIV-1 subtype C ALDSWK and RVLAIERYLKD epitopes with flanking regions were cloned using restriction enzyme tagged PCR products. The recombinants were expressed using the baculovirus expression system.

The insertion of HIV epitopes in the vectors did not significantly alter the protein expression levels. However, the chimeric VP7mu200 and VP7mu177 proteins did not show the
characteristic crystalline structures observed with VP7mu200, VP7mu177 and unmodified VP7 in insect cells. Scanning Electron Microscopy revealed flat, rounded particulate structures with a rough to layered surface. The insertion of 50, 53 and 101 amino acids thus abolishes the smooth hexagonal crystalline formation characteristic of the VP7 protein. This is partly due to steric interference which most likely disrupted the hydrophobic bonds. The structural disruption of the VP7 particle observed with the 50 aa insertion is not further distorted in the 101 aa insert.

Comparative studies show VP7mu200 to be more sensitive to the hydrophobic nature of an insert with regards to particle assembly than VP7mu177. VP7mu177 with and without HIV epitopes assemble into large particles predominantly found in fractions 1-4 at the bottom of a sucrose density gradient. VP7mu200 with a double HIV insert predominantly assembles into large particles (fractions 1-4) and the single HIV inserts assemble into predominantly smaller particles (fractions 5-8). Mechanical lysis of the infected cells containing the chimeric VP7mu200 particles compared to detergent treatment of similarly infected samples reveal possible lipid association suggesting that VP7mu200 may form inclusion bodies. Despite differences in particle assembly between the vectors, both chimeric VP7mu177 and chimeric VP7mu200 particles were shown to be antigenic. Future prospects include conformation and immunology studies pertaining to immunizations with the chimeric proteins, analysis of sera to ascertain if antibodies have been raised as well as neutralization assays to distinguish between the capabilities of the different insertion sites.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acids</td>
</tr>
<tr>
<td>AHSV</td>
<td>African horsesickness virus</td>
</tr>
<tr>
<td>AHSV-9</td>
<td>African horsesickness virus serotype 9</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B cells</td>
<td>Bone marrow derived lymphocytes that produce antibody</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette Guérin</td>
</tr>
<tr>
<td>β₂-m</td>
<td>β₂-microglobulin</td>
</tr>
<tr>
<td>BHK cells</td>
<td>Baby hamster kidney cells</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BTV</td>
<td>Bluetongue virus</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celcius</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CLP</td>
<td>Core-like particle</td>
</tr>
<tr>
<td>cm³</td>
<td>Centimetre cubed</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CSP</td>
<td>Sporozoite coat protein</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Deionized distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DPV</td>
<td>Muscovy duck parvovirus</td>
</tr>
<tr>
<td>ds</td>
<td>Double-stranded</td>
</tr>
<tr>
<td>DTT</td>
<td>1, 4-dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>Exempli gratia (for example)</td>
</tr>
<tr>
<td>ENV</td>
<td>Envelope</td>
</tr>
<tr>
<td>et al.</td>
<td>And others</td>
</tr>
<tr>
<td>Fig.</td>
<td>Figure</td>
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<tr>
<td>gen</td>
<td>Gentamycin</td>
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</table>
GFP    Green fluorescent protein
gp 120  Glycoprotein 120
GST    Glutathione S-transferase
HA     Hemagglutinin
HCl    Hydrochloric acid
hr(s)  Hour(s)
HbcAg  Hepatitis B virus nucleocapsid
HIV    Human Immunodeficiency virus
hpi    Hours post infection
HSV    Herpes simplex virus
i.e.   That is
IFN-α  Interferon alpha
Ig     Immunoglobulin
IL-12  Interleukin 12
IPTG   Isopropyl-β-D-thiogalactopyranoside
IRIV   Immunopotentiating reconstituted influenza virosomes
k      Kilo
kan    Kanamycin
KCl    Potassium chloride
KH₂PO₄  Potassium phosphate
LB broth Luria-Bertani broth
LCMV   Lymphocytic chorio menningitis virus
LSDV   Lumpy skin disease virus
M      Molar
mAb    Monoclonal antibody
MCS    Multiple cloning site
MgCl₂  Magnesium chloride
MHC    Major histocompatibility complex
MgSO₄  Magnesium sulphate
min    Minute/s
ml     Millilitre
mm     Millimetre
mM     Millimolar
MOI   Multiplicity of infection
mRNA   Messenger RNA
mu     Mutant
MVA    Modified Vaccinia Ankara
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Sodium phosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>NICD</td>
<td>National Institute of Communicable Diseases</td>
</tr>
<tr>
<td>NIV</td>
<td>National Institute of Virology</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometres</td>
</tr>
<tr>
<td>NS1, NS2, NS3</td>
<td>Orbivirus nonstructural proteins 1, 2 or 3</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ΦX</td>
<td>PhiX174/Hae marker</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomolar</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sec</td>
<td>Second/s</td>
</tr>
<tr>
<td>Sf9</td>
<td>Spodoptera frugiperda insect cells</td>
</tr>
<tr>
<td>T=1</td>
<td>Triangulation number of T=1</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>T cells</td>
<td>Thymus derived lymphocytes</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptors</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroximethyl)-aminomethane</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>UHQ</td>
<td>Ultra high quality water</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>µg</td>
<td>Micrograms</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitres</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometres</td>
</tr>
<tr>
<td>UP</td>
<td>University of Pretoria</td>
</tr>
<tr>
<td>UNAIDS</td>
<td>Joint United Nations Programme on HIV/AIDS</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>VEE</td>
<td>Venezuelan Equine Encephalitis</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>VP 1 – 7</td>
<td>Virus protein 1 to 7</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume ratio</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume ratio</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>3-D</td>
<td>Three dimensional</td>
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5. Aims

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CONGRESS CONTRIBUTIONS
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Chapter 1
Literature review

Introduction
Vaccination is one of the most important and cost effective ways of protecting animals and humans from infectious diseases. The concept of vaccination has a long history that can be traced as far back as 11th century China where a powder of dried smallpox scabs was inhaled for protection against this dreaded disease. One of the first scientific attempts that demonstrated the power of vaccination can be attributed to Edward Jenner, who in the late 1700’s inoculated a young boy with cowpox pustules, giving the boy protection against subsequent challenge with virulent smallpox. Early methods were crude and performed without any knowledge of immunology and the nature of the infectious agent. Since then, vaccines have successfully been developed against major diseases such as diphtheria, tetanus, poliomyelitis, pertussis, measles, mumps and rubella. Smallpox has been eradicated largely due to the widespread use of the smallpox vaccine (André, 2003).

Vaccination is based on the principle that specific B and/or T cells of an animal are invoked after encountering a pathogen or any foreign antigen, leaving behind memory cell populations that can be clonally expanded upon the next encounter. This memory cell population will remember the foreign antigen long after the vaccination. A second encounter with the specific foreign antigen will induce a rapid and effective immune response compared to the primary response induced by the vaccine via these memory cells. A vaccine therefore refers to any means of presenting foreign proteins of a harmful organism to the host immune system, in order to elicit an appropriate immune response (Roitt et al., 1998).

Effective vaccines should be able to induce an immune response that is appropriate to the pathogen associated with the specific disease, i.e. cell-mediated immunity to control intracellular organisms and antibodies to act against toxins and extracellular organisms (Roitt et al., 1998). Ideally, a vaccine should elicit cross-neutralizing antibodies when multiple strains of an organism exist. The elicited antibodies should not enhance the infection by targeting the host’s immune system. A vaccine is required to induce the immune response at the site of infection and be able to evoke long-lasting immunity. The vaccine preparation itself should be stable, non-toxic, cost-effective and manageable in third world countries where refrigeration and administration infrastructures are often a problem (Levy et al., 1994; Johnson and Kalams, 1998). Not all the vaccines available on the market meet these requirements. There is, therefore, a growing need to improve the safety and efficacy of existing vaccines and to develop new vaccines.
The advances in recombinant DNA technology have greatly improved our means of developing new vaccines. A wide variety of different vaccine strategies are currently being investigated to accomplish this goal. In this study a strategy for presenting HIV-epitopes to the immune system via VP7 particles of African horsesickness virus (AHSV) will be discussed.

1. Traditional vaccines

There are a number of strategies available of which traditional vaccines were the first generation. They consist of whole virulent organism that have either been attenuated or inactivated.

Live vaccines are capable of infecting target cells and replicating in the host. Live attenuated vaccines are based on infectious virus that is passaged through an atypical host animal or cell culture. During this process, mutations occur in cell culture that result in a loss of virulence of the organism (Levy et al., 1994). The success of this approach is exemplified by the worldwide eradication of smallpox using live attenuated vaccinia virus. In another example Calmette and Guérin cultured a bovine strain of *Mycobacterium bovis* which was developed *in vitro* to a less virulent form known as BCG providing protection against TB (Levy et al., 1994; Roitt et al., 1998). Viral virulence can also be decreased or eliminated by deleting the pathogenic genes (Slack et al., 2000).

Inactivated vaccines are traditional vaccines that consist of viruses or bacteria that have lost their infectivity by chemical or mechanical damage of the genome. Chemical or mechanical agents such as formaldehyde and UV radiation are used for inactivation. Many inactivated vaccines have been used with great success against veterinary pathogens such as foot and mouth disease virus. There are, however, many concerns about the safety of inactivated vaccines (Levy et al., 1994; Johnson and Kalams, 1998).

Many live attenuated and inactivated vaccines have serious limitations that restrict their widespread use. A major obstacle is the inability to culture certain pathogens in cell culture, such as Herpes simplex virus (HSV) and Human Immunodeficiency virus (HIV) (Yamanouchi et al., 1998; Amara and Robinson, 2002). Attenuation of pathogens such as HIV will never be accepted in vaccine formulations, considering the danger of reversion back to virulence. Batch reproducibility is not guaranteed as insufficient attenuation or inactivation may occur. Random inactivation of the virus using chemical or mechanical methods may also damage the antigenicity of the preparation.
Vaccine preparations risk being contaminated by other viruses during cell culture (Glick and Pasternak 1998; Levy et al., 1994; Casal, 1999). Traditional vaccines often do not offer full protection against all possible viral serotypes.

The vaccination of infants, the elderly and immuno-compromised individuals may cause illness. In addition, cell culture vaccine production is often expensive. Large scale production is required thereby increasing the cost as yield and production rate is often low. Safety and training of competent laboratory personnel is also required.

Recombinant vaccines are aimed at addressing some of these inherent problems associated with traditional vaccines.

2. Recombinant vaccines
Recombinant vaccines are preparations that are aimed at presenting immunological important proteins or epitopes to the immune system, generated via recombinant DNA technology. Extensive use is made of viral vectors or plasmid constructs for expressing the relevant protein(s). A large variety of vaccine presentation systems are currently being investigated, each with their own advantages and disadvantages. These include the use of live viral vectors, subunit vaccines, peptide vaccines and naked DNA.

Recombinant vaccines have several advantages compared to traditional vaccines. Recombinant vaccines are regarded as safer as subunits of the pathogen are used (Yamanouchi et al., 1998; Glick and Pasternak, 1998). In cases where pathogens cannot be safely attenuated or inactivated, as is the case for HIV, recombinant vaccine strategies are available. Another advantage lies in the fact that individuals vaccinated with recombinant vaccines will only present a subset of antigens of the pathogen (Paoletti, 1996). This allows for a distinction between naturally infected and vaccinated individuals. The distinction is not possible with traditional vaccines since antibodies directed against the whole pathogen will be present when analysed via immunological methods. However, serological tests may be available to distinguish between naturally infected and vaccinated animals but may not be specific and sensitive enough for a diagnosis. Therefore there is a need for improved vaccines for diseases such as African horsesickness virus and foot and mouth disease virus where the distinction between naturally infected and vaccinated animals could greatly benefit the international animal trade.

There are several recombinant vaccine strategies and these will be briefly discussed including their advantages and disadvantages.
2.1 Live recombinant viral vaccines

Promising delivery systems currently being investigated are based on the use of live viral vectors as vaccines. An immunological important foreign gene is integrated into the genome of the viral vector, thereby creating a recombinant viral genome. Viral vectors are selected or manipulated to have a reduced virulence that would be non-pathogenic to the host. Foreign genes usually have to be integrated into non-essential sites of the genome. The viral vector usually presents the foreign protein on the viral particle (Glick and Pasternak 1998; Levy et al., 1994). Vectors include both DNA- and RNA virus based systems.

A South African example of a live recombinant RNA vaccine is the Venezuelan Equine Encephalitis (VEE) replicon (Williamson et al., 2000). The particles have a self-amplifying VEE RNA in which the structural gene was replaced with Gag/Pol/Env HIV-1 subtype C genes. Recombinant VEE replicons expressing Gag DU422 were produced of which vaccine trial preparations are already underway (Van Harmelen et al., 2001). High levels of expression were observed with no VEE capsid present, thus eliminating reproduction and spread to neighbouring cells. It was capable of targeting lymphoid tissue and induced strong cellular and humoral responses, which could be advantageous for lymphoid infecting diseases (Williamson et al., 2000).

The well known live viral recombinant DNA vaccine system is based on the vaccinia virus vector (Moss, 1996). Numerous examples of vaccinia based presentation systems exist in the literature. The rabies glycoprotein G recombinant vaccinia virus used for the oral vaccination of foxes in Europe, raccoons and striped skunks in North America demonstrated its effectiveness in wildlife vaccination (Yamanouchi et al., 1998). Various Gag, Pol, Nef, Tat and Env HIV-1 epitopes have already been expressed in vaccinia, canarypox and Modified Vaccinia Ankara (MVA) based viral vectors and are in various stages of basic research and development of which several of these are currently in human trial phases (Williamson et al., 2000).

A major advantage of the live viral vaccine strategy is that both humoral and cell-mediated immune responses are elicited. The infection mimics a natural infection, aiding in eliciting an immune response against both the foreign protein and the viral vector. The viral vector thus acts as a natural adjuvant for the foreign protein (Roitt et al., 1998). By mimicking a natural infection, the specific site of infection, for example mucosal surfaces, is also exploited by the viral vector, thus targeting the immune response to specific cells in tissues (Levy et al., 1994; Johnson and Kalams, 1998).
The mucosal or systemic immunization of mice with recombinant adenovirus expressing glycoprotein B of the Herpes simplex virus resulted in specific CTL responses limited to the mucosal or systemic lymphoid tissues that were targeted by the vector (Johnson and Kalams, 1998). The large genome of live attenuated antigen display systems have the added advantage of displaying large proteins and can thus be used as multivalent vaccines.

A strong response to viral proteins (of the vector) other than the vaccine antigen will reduce the effectiveness of the vaccine due to efficient clearing of the virus by residual antibodies in the patient. This situation may occur due to a previous vaccination or infection with the viral vector used, thereby rendering the vaccination inefficient (Bona et al., 1998). Possible integration properties of certain viruses are also a cause for concern. Viral proteins that resemble host proteins could induce autoantibodies in the host. For poxvirus vectors, unacceptable virus-related illnesses or side effects may exclude the vector from use in immuno-compromised individuals and the elderly (Moss, 1996). These fears are being addressed by the development of attenuated poxvirus vectors, such as modified vaccinia virus and species-specific poxviruses such as fowlpoxvirus and racoonpoxvirus (Yamanouchi et al., 1998; Moss, 1996; Paoletti, 1996).

2.2 Subunit vaccines

Subunit vaccine strategies are based on using non-replicating preparations for vaccination. Immunological important proteins, peptides or epitopes are expressed via a suitable expression system and purified before being administered as a vaccine. These proteins are often surface proteins that are part of a membrane, envelope or core. Many examples of membrane proteins, virus-like particles (VLPs), core-like particles (CLPs) or individual particulate proteins exist of which a few examples are presented in the following paragraphs. This offers a great degree of safety as live pathogens or vectors are not used which may cause disease.

Herpes simplex virus (HSV-1) is being investigated as a possible subunit vaccine against the respective disease itself as well as an epitope display vector for other diseases using recombinant technology. The immunogenic HSV-1 viral envelope glycoprotein D was modified by the removal of the C-terminal transmembrane binding domain, which is involved in host membrane binding. This allowed the secretion of the soluble protein into the external medium. Laboratory trials reported neutralization success against both HSV-1 and HSV-2 (Glick and Pasternak, 1998).
Immuno-potentiating reconstituted influenza virosomes (IRIVs) is an example of a novel approach to subunit vaccines. IRIV contains influenza surface glycoproteins hemagglutinin (HA) and neuraminidase combined with phospholipids: 70% Lecithin, 20% Cephalin and 10% envelope phospholipids from the selected influenza virus. Epaxal (for protection against hepatitis A) and InflexalV (for protection against influenza) are already on the market (Glück and Metcalfe, 2002). These spherical vesicles provide a natural presentation system for use as an adjuvant. IRIVs can elicit either B or T cell responses. Cellular responses are achieved by encapsulating the antigen within the IRIV. Antibody responses are elicited by surface presentation via integration into the lipid bilayer, anchoring within the lipid bilayer, cross-linking to HA glycoproteins in the bilayer or adsorbing antigens to the membrane of the bilayer (Glück and Metcalfe, 2002).

An example of the core particle vaccine approach is represented by Hepatitis B. The nucleocapsid of Hepatitis B (HbcAg) has been engineered and developed as a particulate vaccine to display foreign epitopes (Loktev et al., 1996; Kratz et al., 1999). HbcAg consists of multiple dimers of one protein that contains T cell epitopes. Expression of this protein results in the formation of HbcAg core particles (Street et al., 1999). A green fluorescent protein (GFP) insert of 238 amino acids has been successfully displayed on the surface of these HbcAg core particles (Kratz et al., 1999). B cell epitope insertions of the human papillomavirus results in powerful antibody responses using these CLPs. However, insertion of CTL epitopes such as the E7 protein from the human papillomavirus results in an increase in hydrophobicity which buries the peptide in the particle due to the hydrophobic nature of CTL epitopes. It has also been reported that some T cell epitope inserts caused the formation of insoluble material or destabilised the core structure due to its hydrophobic nature (Street et al., 1999). Loktev et al. (1996) have resolved this problem with the production of mosaic HbcAg core particles. These particles contain both native wild type HbcAg core protein and recombinant HbcAg containing a Venezuelan equine encephalomyelitis, VEE epitope. Previous expression of the recombinant protein containing only VEE resulted in insolubility with no core assembly. Co-expression of wildtype and recombinant proteins form mosaic particles that stabilize the core structure (Loktev et al., 1996). Other HbcAg particles have been produced using a different insert. Chimeric HbcAg particles containing circumsporozoite protein (sporozoite coat protein CSP, 326-345 aa) and CSP repeat sequences from *Plasmodium berghei* and *P. yoelii* elicited high titered long lasting antisporozoite antibodies in mice. This is a promising result for a pre-erythrocytic stage vaccine in the search for a *Plasmodium falciparum* malaria vaccine. The CSP 326-345 aa region contain overlapping CD4⁺ and CD8⁺ T cell epitopes while the CSP repeat sequences elicit the antisporozoite antibodies (Sällberg et al., 2002).
Parvovirus VP2 particles consist of a single immunogenic capsid protein that was modified to serve as a particulate vaccine (Casal, 1999; Rueda et al., 1999). The immunogenic VP2 is the major component of the parvovirus capsid (Casal, 1999). VLPs were obtained by allowing VP2 to self assemble. The N terminus can be used to display CD4+ and CD8+ epitopes while a loop region is used for B cell epitopes, thus eliciting both cellular and humoral immunity. Insertion of the poliovirus C3:B epitope in amino acid positions 226, 227 and 228 resulted in strong neutralization antibody responses (Rueda et al., 1999). The use of single protein VP2 in VLPs is advantageous as a high degree of VLP stability is achieved, is highly immunogenic and can be easily purified in large amounts (Casal, 1999; Rueda et al., 1999). In Muscovy duck parvovirus (DPV), the VP2 and VP3 capsid proteins self assemble to form empty VLPs using the baculovirus expression system. These VLPs are morphologically and antigenically identical to the native parvovirus virions. They were shown to elicit DPV neutralizing antibodies with titers consistent with those achieved by commercial attenuated vaccines (Le Gall-Reculé et al., 1996). Another example of a subunit particle approach is the development of African horsesickness virus VP7 and NS1 for antigen display. This will be discussed in more detail in section 4.3.2.2.

The advantage of a subunit vaccine lies in the use of purified protein which ensures the stability and safety of the product as the vaccine components are known and free of contaminating proteins that may cause side effects (Glick and Pasternak, 1998). Compared to inactivated vaccines which are also purified from cellular material, subunit proteins are only a subset of viral proteins from the virus where an inactivated vaccine contains all the viral proteins. The use of a subunit vaccine eliminates exposure to pathogenic genes as they are not contained in the preparation. In some cases the purification is relatively simple and cost effective. Although subunit vaccines were initially believed to only stimulate a humoral immune response, various examples have illustrated that cell-mediated immunity is indeed possible (Casal, 1999). Constructs can also be readily changed when problems occur such as when insufficient immunogenicity is observed. Immunogenicity can then be increased by coupling the protein to an antibody that recognizes cell surface determinants (immunotargeting) (Cook and Barber, 1997). Another method is to attach T cell epitopes to ligands of the receptors, expressed on antigen presenting cells or T cells that would result in internalization, processing, and presentation with MHCII for CD4 presentation (called cell targeting). An example of which is HIV envelope peptide coupled to β2-m, which was efficiently internalized by antigen presenting cells via their membrane receptors. However production problems such as batch reproducibility and formations of aggregates curb the use of receptor-linked peptide delivery (Bona et al., 1998).
Disadvantages of subunit vaccines are that they often require the use of adjuvants and more than one administration to offer an effective and long-lasting immune response (Casal, 1999).

Some purification methods are costly and may alter the protein conformation (Glick and Pasternak, 1998). The antigenicity of the foreign epitope can be affected by conformation and cell type used for production causing loss of immunogenicity. Some subunit vaccines do not induce endogenous synthesis of viral proteins in antigen presenting cells and are thus not able to stimulate CD8+ CTL responses for cellular immunity (Johnson and Kalams, 1998).

2.3 Peptide vaccines
Specific immunogenic sequences of a virus are selected to synthetically produce small peptides (15-20 amino acids long) for use as vaccines. It is one of the safest vaccines available as it is very specific and offers good quality control (Levy et al., 1994).

Problems often arise when peptides are less than 20 aa in length as they are generally poorly immunogenic and need to be administered with a larger protein or adjuvant (Levy et al., 1994). They also risk eliciting narrow immune responses (Johnson and Kalams, 1998). The epitope must also be contiguous with single epitope repeats rarely sufficiently immunogenic. A prerequisite of eliciting antibodies are that within the short sequence used, the peptide must be able to mimic its native conformation (Glick and Pasternak, 1998). The lack of 3D conformation in synthetic peptides also makes them vulnerable to degradation. Bigger flanking regions can be incorporated but in a dangerous pathogen these regions may contain sequences that are immunosuppressive or stimulate autoimmunity. Efforts have been made to increase the immunogenicity of peptide constructs with the production of so-called hyper variable constructs and using receptor linked cell targeting. Production problems such as batch reproducibility and aggregate formations curb the use of receptor linked peptide delivery. Other methods to enhance immunogenicity include circularization of peptides by introducing sulphide bonds, fusion of B cell epitopes, and fusion of T cell to B cell epitopes or expression in a live virus vector or subunit vaccine vector (Hewer and Meyer, 2002; Loktev et al., 1996).

2.4 Naked DNA vaccines
Naked DNA vaccines consist of genetic material that encodes proteins cloned into plasmid expression vectors which elicits humoral and cellular immune responses (Slack et al., 2000). These plasmids feature strong promoters, an origin of replication, antibiotic resistance, and polyadenylation signals. Vector (CpG) dinucleotides with flanking 2 x 5' purines and 2 x 3'
pyrimidines have also been added which stimulate macrophages, dendritic cells, B cells, T cells and cytokines such as IL-12, TNF-α and IFN-α via dendritic cells thereby enhancing the immune response (Guranathan et al., 2000).

An advantage of naked DNA is the large-scale production of highly purified products at low cost. Bacterial plasmids do not replicate in mammals and are thus considered safer compared to other vaccine systems. Vaccines against dangerous pathogens can be made using subsets of genes cloned into vectors. Thus research can continue in institutions that do not have the necessary safety clearance to work with the particular organism as a whole. As the preparations only include plasmid DNA, young animals and multiple vaccinations should not stimulate the immune response against the vector DNA and cause adverse reactions. The preparations are also stable and cold storage is not a requirement (Yamanouchi et al., 1998).

Even though naked DNA displays some of the requirements of an ideal vaccine, it also has its disadvantages. Plasmid vectors with strong expression do not always guarantee an immune response to the antigen (Yamanouchi et al., 1998). Good immunogenic responses may require numerous doses or need to be boosted with other live expression vectors. This increases the cost and research involved.

3. HIV
3.1 Introduction
HIV is a perfect example of a disease for which limitations exist on vaccinology. Inactivated and attenuated vaccines present a health risk to individuals and will never be accepted. Recombinant vaccines offer safer alternatives and offer a distinction between naturally infected and vaccinated individuals. HIV is the scourge of humanity today and is the focus of numerous research projects.

3.1.1 Epidemiology in South Africa
In the year 2010 ± 500 000 AIDS related deaths are predicted to occur. The people most at risk are aged between 15-26 (women) and 15-32 (men) years. The infection rate in women is higher than men with young women, migrant workers, and sex workers the most at risk (Williams et al., 2000). At the end of 2001 UNAIDS WHO, national governments, and research institutions calculated that 5 million people were infected in South Africa. The total comprised of 4.7 million adults (men and women aged 15-49); 250 000 infected children and with AIDS related deaths numbering 360 000 which would result in 660 000 AIDS orphans under the age of 15 years (UNAIDS/WHO, 2002). The high-risk groups contain the most
productive individuals. These statistics have implications for the economy and birth rate of a
nation. Thus strategies need to be implemented to control the number of new infections in
these groups.

3.1.2 Addressing the problem in South Africa

3.1.2.1 Education and antiretrovirals
The main anti-AIDS strategies have been based on awareness, safe sex campaigns and the
use of antiretroviral drugs (Harrison et al., 2000). The use of antiretrovirals has been
beneficial in prolonging and extending the quality of life for numerous AIDS patients.
However problems have been reported with antiretroviral use such as: cost, significant side-
effects, toxicity as well as the logistics of administration and monitoring drug therapy, putting
them above the means of most developing countries (Kuritzkes, 2000; Roitt et al., 1998;
Webber et al., 2000; Rosen et al., 2000). The cost effective alternative to antiretrovirals, in
developing countries, would be vaccination.

3.1.2.2 Vaccines as an alternative form of disease management
Vaccination is more cost effective than medication. Theoretically an ideal vaccine will be a
single treatment. This will make it possible for individuals who could not afford drug therapy
to receive treatment. Vaccines may have fewer side effects than drugs, thus improving the
quality of life and increasing the productive span in the individual's life. An ideal vaccine
would prevent HIV infection thus decreasing the number of AIDS sufferers in the long run.
However, a good vaccine would prevent AIDS progression making HIV infection a chronic
but manageable disease.

Most vaccine initiatives underway in America and Europe focus on HIV subtype B. It is
therefore crucial for countries in which other subtypes dominate to develop their own vaccine
strategies based on their subtype prevalence. As the majority of HIV research has been done
in America and Europe, more is known about subtype B than subtype C (Morris et al., 2000).
Subtype C viruses seem to be sensitive to inhibition by agents that bind the gp120/gp41
complex such as 2F5, IgGb12 and 2G12 antibodies (Morris et al., 2000). Subtype C
sensitivity to 2F5 is thus an attractive option available for vaccine development in South
Africa.

3.2 Basic molecular biology

3.2.1 Introduction
HIV is a retrovirus that belongs to the genus Lentivirinae. It is transmitted by blood, sexual
fluids and from mother to child during birth. It is characterized by low levels of CD4 + and high
levels of CD8+ T lymphocytes in infected individuals (Levy et al., 1994). Patients are vulnerable to secondary infections such as Herpes and usually die of opportunistic infections.

3.2.2 HIV proteins

HIV-1 viral particles contain a nucleoprotein core within a lipid bilayer comprised of Env proteins gp120 and gp41. The genome consists of 2 identical positive-sense single stranded RNA molecules that are associated with tRNAs, mature Gag and Pol proteins (Leigh Brown and Holmes, 1994; Hope and Trono, 1998).

At least 9 proteins are encoded by the genome:
1) The major structural proteins: Gag, Pol, Env;
2) The regulatory proteins: Tat, Rev and

Mature Pol proteins consist of the following viral associated enzymes: Protease, Reverse Transcriptase, and Integrase. HIV-1 virions also contain viral proteins Vpr, Vif, and Nef. Tat interacts with viral long terminal repeats for up-regulation of viral replication. Rev prevents splicing of large viral mRNAs during transport to the cytoplasm for expression. Nef controls the viral replication rate (Hope and Trono, 1998; Levy et al., 1994). A high degree of variability exists in the Env, Tat, Rev and Nef proteins. This in turn limits their use in vaccine strategies (Young, 1997).

3.2.3 HIV expression

Firstly reverse transcription generates cDNA from the viral RNA genome. Reverse transcriptase has poor fidelity causing mutations in the genome, which include amino acid substitutions, frame shifts, and deletions. Recombination also takes place during cDNA synthesis as template switching can occur between two viral RNA molecules. This variation creates diverse viruses with differences in cell tropism and drug resistance (Young, 1997). Cell tropism may also be due to amino acid differences in the V3 loop of HIV-1 gp120 (Premack and Schall, 1996). cDNA flanked by long terminal repeat sequences, is integrated into the host cell chromosomal DNA forming a provirus (Leigh Brown and Holmes, 1994). The host cell transcription machinery is then used to express the HIV viral proteins (Young, 1997).

HIV-1 expression requires elements of the host cell transcription machinery such as RNA polymerase, transcription factors Sp1 and NFκB and the HIV regulatory proteins Tat and Rev. Unspliced genome length viral RNA transcripts are packaged into the HIV viral cores. Env proteins are synthesized in the Endoplasmic reticulum and transported by the host cell secretory pathway. Gp160 is cleaved in the Golgi-apparatus by cellular proteinase to
generate gp120 and gp41 before being transported to the cell surface. Gag and Gag-Pol polyproteins are cleaved by HIV viral proteases to generate mature viral particles (Young, 1997).

### 3.2.4 HIV transmission

The Env proteins of the mature viral particles bind to CD4 on the cell surface of CD4⁺ T lymphocytes, which are involved in the immune response against foreign antigens (Roitt et al., 1998). Infection and spread of HIV occurs via mucosal routes. HIV is transported to the lymph nodes via Dendritic and Langerhans cells (Gray and Puren, 2000; Williamson et al., 2000). As HIV infects cells associated with the immune response, it can be expected that the immune response will be impaired. Vaccine design has focussed in the past on the envelope of HIV gp120/gp160. These proteins are exposed on the viral surface and should theoretically be accessible to the immune system.

Unfortunately not all of the proteins exposed on the surface can effectively interact with the immune system. Problems occur when epitopes on gp120 and gp41 are hidden from the immune system by subunit interactions, conformations, positions of variable loops and N-linked glycan moieties on the gp120 surface (Montefiori and Evans, 1999). Thus for some of these potential epitopes to elicit an immune response, they need to be isolated from their “restrictive” environment for effective presentation. The next paragraph discusses gp41 in more detail.

### 3.2.5 HIV gp41

Gp41 consists of an extracellular domain, a hydrophobic transmembrane region and a cytoplasmic domain as illustrated in figure 1.1.

![Figure 1.1](image-url)

Figure 1.1: A schematic representation of gp41 based on the HIV-1 LAI amino acid sequence with the ecto (extracellular) and endodomain (intracellular), RILAVERYLKD (Rila) and ELDKVAS (Eld) epitopes indicated. This figure was combined from figures published by Wild et al., 1994 and Sackett and Shai, 2002.
The gp41 pre-transmembrane sequence may be a signal for lipid domain targeting and also anchors the gp120-gp41 complex to the host membrane (Sáez-Cirión et al., 2002).

The cytoplasmic domain contains 2 amphipatic α-helices, a leucine zipper motif and determinants that promote Env internalization (Kim et al., 2002; Sackett and Shai, 2002; Kao et al., 2001; Chen, 1994; Jiang et al., 1998). The leucine zipper motif is critical to virus entry (Wild et al., 1994). The noteworthy epitopes for vaccine development are located on the ectodomain that will be described below.

The gp41 extracellular/ectodomain consists of 2 α-helical regions. One of these helical regions is adjacent to the N terminal fusion peptide and the other is near the C terminal transmembrane region. There is also a disulphide bonded loop region in the ectodomain, which may act as a hinge in the formation of the trimer of hairpins involved in fusion. The fusion peptide of gp41 in its non-fusogenic state consists of disordered forms that during fusion transforms into a helix for insertion into the host membrane. This fusion peptide in its non-fusogenic form is located within the envelope. Binding of gp120 to CD4 results in gp41 adopting a more stable conformation with the fusion peptide projected away from the viral membrane into the host membrane. During fusion specific helical regions of gp41 interact with one another creating a hairpin structure, which spans both the viral, and host membranes (Labrosse et al., 1997; Blair et al., 2000). Gp41 anchors its hydrophobic N terminal fusion peptides into the host membrane for fusion(Siebert and Hummer, 2002; Follis et al., 2002). This makes gp41’s involvement in membrane fusion a prime target for vaccine development.

3.3 HIV vaccines
3.3.1 The ideal HIV vaccine
An HIV vaccine should aim to block the development of AIDS upon infection with HIV, lower the viral load, decrease the probability of transmission and increase the CD4+ T cell count (Carlos et al., 2000). HIV specific CTL pressure may lead to selection of viral escape mutants, thus immunization should elicit broad CTL responses. CD8+ modified epitope escape can occur via antigen processing and peptide transport which have been affected by mutations in flanking regions. Altered peptide binding to MHC can occur due to mutations in anchor residues. Mutations in these regions can also affect T cell receptor recognition (Mortara et al., 1998). The vaccine should also be reactive across multiple subtypes and isolates and not impair the immune system. Additional requirements have already been discussed.
3.3.2 First generation recombinant monomeric gp120 - and gp160 - based vaccines.
A major problem of recombinant monomeric gp120 - and gp160 - based vaccines is their inability to elicit CTL responses as they are unable to enter the MHC I pathway for processing and presentation of antigen. Another problem is that neutralizing antibodies that have been elicited in the past inhibit laboratory HIV strains and not primary strains (Williamson et al., 2000). Difficulties encountered by antibodies include the high degree of glycosylation, which reduces antibody accessibility to gp120. Further reduction of available protein surface is due to the heterodimeric interaction of gp120 with gp41 as well as the trimerization of gp41 and gp120 respectively (Parren et al., 1998). The CD4 binding site is found in a pocket and is poorly accessible to antibodies. Conformational changes due to binding only expose antibody surfaces for a limited time period (Sattenau et al., 1999).

Sequence variation in the major neutralizing and CTL epitopes causes problems in vaccine design as broadly reactive and long lasting cellular and humoral responses are not achieved. Thus mixtures containing all the hypervariable regions of gp120 are needed to circumvent this problem (Carlos et al., 2000). That is an unrealistic expectation of any vaccine system. The emphasis on proteins used in vaccines has thus moved from monomeric Env units to viral subunits. Smaller immunogenic viral units are being investigated to circumvent the above problems. Multiple isolate vaccines can be created thus focussing the immune response to important regions which eliminates the incorporation of immunopathogenic or immunosuppressive proteins (Hanke et al., 1999).

3.3.3 Recent vaccine strategies
HIV vaccines have been targeting virus entry and replication. Vaccines eliciting CD8+ cells have also become more popular as they kill infected cells and suppress viral replication. Research has identified numerous T cell epitopes specific to various proteins with intra and intersubtype conservation, with various vaccine strategies being investigated (Amara and Robinson, 2002; Bojak et al., 2002).

Adjuvanted DNA vaccines, DNA-primed live virus vector boosted vaccines and live virus vector primed and boosted vaccines have been made with genes such as Simian Immunodeficiency virus (SIV) Gag-Pol and HIV Envs 89.6 or 89.6P as antigens. Dendritic cell targeting has been employed to enhance priming of T cells as well as various adjuvants and DNA coated microspheres. Vaccine designs have moved away from large inserts as these make the vectors unstable due to the existence of size limitations in some vectors (Amara and Robinson, 2002; Bojak et al., 2002).
3.3.4 Epitope based vaccines

Epitope based vaccines have become increasingly popular as they consist of defined immunogenic viral information. Multiple isolate representations can be created with conserved epitopes across isolates. The amount of interfering flanking regions is also reduced. These flanking regions may obscure the epitope in its native form, which is responsible for the natural weak neutralization antibody response seen in AIDS patients (Zwick et al., 2001). Isolation of the epitope from these regions may improve the immunogenicity of the epitope by increasing the chance that the foreign epitope will be recognized by the immune system. This will then theoretically increase the amount of neutralization antibodies. Epitopes are defined immunogenic regions which would not include viral sequences that may cause immune dysfunctions. Epitopes can be used in a variety of ways such as in a vaccine expression vector (live vaccine vectors), peptide vaccines and subunit or particulate systems.

3.3.4.1 HIV epitopes and monoclonal antibodies (mAbs)

Monoclonal antibodies that recognize conserved HIV epitopes with a therapeutic value are few (Parker et al., 2001). Three mAbs 2F5, 2G12 and IgG1b12 are known to have significant cross reactive anti-viral activities (Liang et al., 1999). Neutralization targets such as the V3 loop, CD4-binding site and the V2 epitope have also been identified (Scanlan et al., 2002; Ding et al., 2000).

The ELDKWA epitope (located in the C - terminal region of the gp41 ectodomain) has been shown to induce protective responses. ELDKWA is relatively conserved and recognized by the monoclonal antibody 2F5. 2F5 shows neutralization activity against HIV-1 isolates from Africa, Asia, America, and Europe (subtypes A, B and E) (Lu et al., 2000; Brown et al., 1995). 2F5 may function by inhibiting the formation of the six-bundle structure required for membrane fusion (Follis et al., 2002). The gp41 C - terminal has a tryptophan-rich region present in some strains that is critical for cell fusion and thus infectivity. 2F5 contains 3 tryptophan residues from the region that may play a role in its activity (Parker et al., 2001).

It is supported by the observation that 2F5 activity is abolished by tryptophan 673 mutation (Neurath et al., 1995). 2F5 is an IgG isotype and is therefore expected to cross the placenta, thus theoretically being able to block mother to child transmission (Li et al., 1998; Dong et al., 2001). 2F5 is capable of neutralizing virus after short exposure, suggesting a high avidity for its target. The LDKW core of the ELDKWA epitope is present among 4 of the HIV subtypes making it relatively conserved (Conley et al., 1994; Lu et al., 2000). 2F5 inhibits gp41 binding to T cells, B cells and monocytes and prevents the initial infection of mucosal Dendritic cells, thus interrupting transmission to T cells. An ELDKWA tetramer epitope vaccine [C-
(ELDKWAG)_n –BSA] has induced a 1:12 800 – 25 600 antibody response in mice with experiments indicating a 90 % virus neutralization rate (Xiao et al., 2000; Ding et al., 2000). Apart from this gp41 C - terminal epitope, an N - terminal epitope was also discovered.

The RILAVERYLKD epitope is located on the N domain of gp41 and has been shown to be an immunosuppressive domain that may be associated with immunosuppression seen in HIV-1 patients. Studies have indicated that gp41 and type1 interferons have sequence similarity in the RILAV_YLK D motif in their receptor binding regions. This suggests an analogous protective activity (Lu et al., 2000; Brown et al., 1995). RILAVERYLKD is a CD8⁺ CTL epitope (HLA A24 restricted) encompassing the YLKDQQLL residues or may be CD4⁺ T cell HLA DPw4.2 restricted (Chen et al., 1992; Hammond et al., 1991). It is part of the epitope cluster I that is the best exposed on the surface of HIV-1 virions and is located in the hydrophilic region that includes a disulfide loop (Nyambi et al., 2000). It is also located within DP107, which is predicted to have a leucine zipper region (Chen et al., 1995).

The need for improved vaccines is obvious especially with the problems experienced with developing HIV vaccines. Therefore we are interested in developing particulate vaccine systems based on the use of specific AHSV proteins.

4. African horsesickness virus
4.1 Introduction

AHSV infects horses, mules, donkeys and zebra; is transmitted by midges (Culicoides spp.), and is endemic to Sub-Saharan Africa. AHSV outbreaks in horses have economic implications for international horse trade (Venter et al., 1995). The mortality rate is between 70 - 95% in horses, 50% in mules and 10% in donkeys. Four clinical forms exist: a pulmonary form, cardiac form, a pulmonary and cardiac mix and a mild form. The virus affects endothelial cells and monocyte macrophages. Studies suggest that lymphoid organs and lungs are the primary sites of replication (Brown and Dardiri, 1990; Burrage and Laegreid, 1994; Gómez-Villamandos et al., 1999). AHSV is taxonomically defined to the Orbivirus genus of the Reoviridae family with 9 serotypes having been identified to date (Martínez – Torrecaudrada et al., 2001).

The current vaccines available are based on live attenuated and inactivated vaccine strategies. The inactivated vaccine is popular as the risks involved with live vaccines are eliminated. However, insufficient inactivation can be hazardous (Martínez – Torrecaudrada et al., 1996). Interference has been observed in polyvalent live attenuated vaccines during virus replication due to the use of several vaccine strains that results in incomplete immunity.
Another problem concerns AHSV’s potential for gene segment reassortment between strains and serotypes. Single viral serotype vaccines do not offer protection against heterologous serotypes while heterologous serotype protection is a necessity in the field. High doses of live vaccines can cause disease in humans and horses (Roy et al., 1996; Wade-Evans et al., 1998). Antibodies are elicited against all proteins making a distinction between vaccinated and infected horses difficult. (Wade-Evans et al., 1998).

4.2 Molecular biology

AHSV virions have a double-layered protein coat with icosahedral symmetry. The virions contain 10 double stranded RNA segments coding for structural proteins VP1-VP7 and non-structural proteins NS1, NS2, NS3 and NS3A. The outer capsid consists of proteins VP2 and VP5. The inner icosahedral core capsid consists of VP3, VP7 and encloses the minor proteins VP1, VP4, and VP6 with the dsRNA genome. VP2 is a viral haemaglutinin involved in host cell attachment. It is the most variable protein and determines the serotype-specific immune response against the virus. Both VP2 and VP5 are targeted by the host’s neutralizing antibody responses (Williams et al., 1998; Martínez – Torrecaudrada et al., 2001). VP1 is the presumed viral polymerase. VP4 is a guanylyltransferase (viral mRNA capping enzyme). VP6 associates with both single and double stranded RNA and may act as a chaperone in the incorporation of RNA into particles. It may also act as a helicase. VP3 forms the scaffold of the inner core onto which VP7 arranges, is the most conserved protein in the virion and also contains group specific antigenic determinants (Roy et al., 1994; Roy et al., 1996; Kar and Roy, 2003). NS1 forms tubular structures associated with AHSV infected cells. NS2 is a phosphoprotein and has a role in packaging the genome (Grubman and Lewis, 1992; Kar and Roy, 2003). Virulence is associated with NS3 which is cytotoxic and associated with membrane damage during viral release from infected cells (van Niekerk et al., 2001).

4.3 AHSV particulate vaccine delivery systems

4.3.1 Biochemistry of VP7

Bluetongue virus is the prototype Orbivirus. AHSV is similarly an arthropod-borne orbivirus which shares its epidemiology with the Culicoides midge. It is also an economically important virus of ruminants eg. sheep and cattle (Oldfield et al., 1990; Hewat et al., 1992). BTV has been studied in detail and has increased understanding of related Orbiviruses such as AHSV and particularly VP7, which is conserved in related Orbiviruses (Basak et al., 1996). The outer core consists of globular VP5 positioned on 6 membered rings of the inner core VP7 trimers. VP2 appears as sail-shaped spikes that form 60 triskelion type motifs. Within the outer core lies the inner core composed of VP7 and VP3 (Hewat et al., 1992; Roy, 1992).
VP3 is more conserved in sequence and structure as it is involved in interactions with VP7 and VP1, VP4, VP6 and probably with genomic RNA as well and is located on the inside of the inner core particle. VP3 is synthesized first providing the scaffold for VP7 attachment (Loudon and Roy, 1991; Liu et al., 1992; Roy, 1996).

Both BTV and AHSV VP7 have 349 amino acids, is encoded by segment 7 and hydrophobic. However, AHSV VP7 is more hydrophobic compared to BTV (Basak et al., 1996; Roy et al., 1991; Williams et al., 1998; Maree, 2000). Amino acid residues have been identified in AHSV VP7, which may increase the solubility of this hydrophobic protein such as Ala-167 and Phe-209 (Basak et al., 1996; Monastyrskaya et al., 1997). Both AHSV and BTV VP7 are group specific antigens eliciting high titered antibodies (Burroughs et al., 1994).

BTV VP7 has at least 2 epitopes exposed on the virion surface (Lewis and Grubman, 1990; Li and Yang, 1990). The BTV epitopes are defined by the aa sequences of LTRAIARAAYV (339-349 aa) and ARQPYGFFLETEEYQPG (122-139 aa). Both epitopes are linear and continuous (Li and Yang, 1990). A third epitope represented by aa 263-267 is found to be cross-reactive to Epizootic hemorrhagic disease virus (Wilson et al., 2000). In total BTV VP7 has at least 6 distinct antigenic determinants of which at least one conformational and one linear epitope is conserved between BTV and AHSV. This is not surprising as the first 102 aa on the N terminus is highly conserved among VP7 proteins of different Orbiviruses (Yamakawa and Furuuchi, 2001). Two AHSV VP7 mAbs have been identified with at least one antigenically dominant site present. The 5G5 mAb binds a conformational epitope. The 3D2 mAb seems to bind a non-conformational epitope with poor recognition of the native virus (Laviada et al., 1992).

BTV and AHSV VP7 contain an RGD motif (located in the top domain), which is responsible for Culicoides cell binding activity (Yamakawa et al., 1999; Tan et al., 2001). BTV VP7 contains 1 lysine residue and is rich in alanine, methionine and proline (Roy, 1989). In AHSV this Lysine (K255) is replaced with arginine and may have a structural role (Roy, 1992; LeBlois and Roy, 1993). BTV K255 is located on a hinged region between the top and bottom domain and is critical for trimerization folding and assembly into CLPs (Roy et al., 1997; Monastyrskaya et al., 1997; Limn et al., 2000).

The top domain stretches from 121-249 aa. The bottom domain encompasses 1-120 aa and 250-349 aa. The domains of VP7 are twisted so that the top domain of one VP7 molecule is over the lower domain of an adjacent VP7 molecule. A short C terminal arm may tie the trimers together during capsid formation as a 50 aa deletion in this region leads to BTV CLP
abolishment (Roy et al., 1997; Monastyrskaya et al., 1997). The AHSV top domain may be responsible for its insolubility with the Ala-167 residue contributing the most (Williams et al., 1998). Two hundred and sixty (260) triangular spikes of BTV and AHSV VP7 molecules interact with 120 molecules of VP3 to form the CLP as an icosahedral structure. Both the trimers of BTV and AHSV VP7 appear as tripod-like structures with distinct upper and lower domains (Williams et al., 1998).

VP7 molecules are arranged as trimers in a T=13 icosahedral lattice (Basak et al., 1992). The icosahedral lattice consists of 5 quasiequivalent trimers referred to as: P, Q, R, S, and T. These refer to subtle differences in the VP7 trimer conformations in relation to each other to complete the T=13 shell. BTV VP7 can form trimers, pentamers and hexamers in solution (Limn and Roy, 2003). AHSV VP7 form unique disc-shaped crystals when expressed via recombinant baculovirus in insect cells and AHSV infected BHK cells (Chuma et al., 1992). The characteristic crystals formed by AHSV VP7 consist of trimeric molecules containing three monomeric VP7 subunits referred to as A, B and C (Basak et al., 1996). The hexagonal forms of the AHSV VP7 crystals are reminiscent of the segmented ring-shaped capsomers visible on the core particle surface (Burroughs et al., 1994). These crystalline structures have been observed with maximum dimensions of 6 µm and a diameter of 8 µm (Burroughs et al., 1994; Basak et al., 1996).

Interactions between VP7 trimers are non-specific and hydrophobic. The VP7 lattice formation requires the exact fit of 260 trimers (Limn et al., 2000). The top and bottom domains are bound by hydrophobic and specific hydrogen bonds (Roy, 1996).

VP7-VP3 contact occurs via hydrophobic residues present on the flat underside of the trimer (Limn and Roy, 2003). Amino acids in the top domain may be involved in viral receptor interactions (Wilson et al., 2000). The bottom domain controls trimer association into hexamers or pentamers on the lattice. Deletion of the bottom domain may cause altered protein folding, no trimerization or altered VP7-VP3 interactions (Roy, 1996). Channels are located between the hexameric and pentameric VP7 sheets. These are channels for exporting or importing metabolites to internal sites of viral mRNA transcription or release of nascent mRNA from the cores (Hewat et al., 1992; Roy et al., 1997). BTV and AHSV CLP formation is not dependent on the presence of minor core proteins, non-structural proteins, or dsRNA (Roy et al., 1990; Maree, 2000).
VP7 domain switching between BTV and AHSV VP7 did not abrogate CLP assembly. In BTV VP7 M30→R, R22→E and N38→D amino acid changes result in a decreased attachment of VP7 to the VP3 scaffold. P338 and A346 are also important residues in lateral trimer packing on VP3. It is suggested that multiple sheets of VP7 form at different sites initiating assembly with a second set consisting of weaker interactions that fill the gaps between the initial sites. This is supported by mutations in VP7 that still allow some VP7 lattice assembly on VP3 even though the CLP is unstable (Limn and Roy, 2003). From BTV truncation mutants it was concluded that the C terminus is important for CLP formation as it is involved in intra/intermolecular interactions for protein function in CLP formation (Roy, 1996). A loss of charge at aa 318 results in less intact trimers on CLPs. Mutations at R111 result in extremely unstable CLPs (Limn et al., 2000). This information is important when designing CLP based vaccines.

4.3.2 Particulate vaccines

In both AHSV and BTV several vaccine designs are being explored. Some are based on a single particle such as NS1 and VP7 or multiple proteins such as CLPs which involve VP3 and VP7. The VLP approach involves VP2, VP5, VP3 and VP7.

4.3.2.1 NS1

NS1 tubule presence is a characteristic of Orbiviral infected cells. Tubules are mainly located in peri-and juxtanuclear locations within the cell (Monastyrskaya et al., 1994). The tubules seem to be attached to the intermediate filaments of the cell’s cytoskeleton (Eaton et al., 1988; Urakawa and Roy, 1988). They may be involved in maturation of virus particles or may act as chaperones for core assembly until VP1, 4 and 6 and/or the RNA genome has been incorporated (Brookes et al., 1993; Maree and Huismans, 1997). NS1 expressed in baculovirus expression systems also produce these tubular structures. BTV NS1 is a globular protein 68 kDa in size. The NS1 molecule has a diameter of ± 68 nm. The filaments are arranged in a fishnet-like manner. Non-covalent interactions are present between NS1 monomers with some divalent metal cation interactions. The horizontal association of molecules is governed by hydrophobic interactions. BTV NS1 is pH sensitive between 4 and 6.5. NS1 is solubilized at a pH of 9.5 and higher.

Tubules are destroyed by anionic, cationic and zwitterionic detergents but remain intact with non-ionic detergents (Marshall et al., 1990; Maree and Huismans, 1997; van Staden et al., 1998). Reducing agents (100mM dithiothreitol) have no effect. Temperatures beyond 50°C affect tubules. Temperatures at 4°C and below result in filamentous BTV NS1 tubules (Marshall et al., 1990; Maree and Huismans, 1997; van Staden et al., 1998).
Chelators such as EDTA and sonication result in transverse breaking of BTV tubules. Vortexing does not affect tubules (Marshall et al., 1990; Maree and Huismans, 1997; van Staden et al., 1998).

AHSV tubules differ from BTV NS1 tubules in size and weave pattern being 23±2 nm in diameter with lengths up to 4 μm. The tubules exhibit a “cross-weave” pattern with alternating electron dense and less dense regions. Data suggests that AHSV tubules are more fragile than BTV tubules as they are easily disrupted by high ionic strength and pH changes than BTV. Both AHSV and BTV NS1 are resistant to low pH (Maree, 2000).

The AHSV and BTV NS1 tubules are both being investigated for antigen presentation. Two antigenic sites were identified: one from the amino terminal region and one at the C terminus of NS1 BTV and AHSV. This antigenic site is exposed on the surface and could theoretically incorporate large foreign sequences and present them to the immune system. As the tubules are helical it is possible to incorporate larger sequences than with CLPs or VLPs that have protein constraints on the insert size (Ghosh et al., 2002a). This has resulted in investigations into the use of AHSV NS1 as a particulate antigen delivery system (Maree, 2000).

A fusion product with the complete Green fluorescent protein formed BTV NS1 tubules (Ghosh et al., 2002a). Another fusion product with a CD8+ T cell epitope from Lymphocytic chorio meninigitisvirus (LCMV) nucleoprotein induced strong protective immune responses in mice (Ghosh et al., 2002a). NS1 C – terminal extension mutants were constructed using PCR amplified hepatitis B virus preS2 region (48 aa), Clostridium difficile toxin A fragment (48 aa) and bovine leukaemia virus p15 matrix protein (109 aa) products. These mutants each formed highly immunogenic tubular structures (Mikhailov et al., 1996).

In another investigation a unique XhoI site in the C terminal hydrophilic region of AHSV NS1 allowed the insertion of VP2 epitopes in AHSV-9 NS1. AHSV VP2 elicits protective serotype specific neutralizing antibodies in mice. Two insertions were made with one corresponding to 40 aa (364-404 aa) and another to 150 aa (270-420 aa) of VP2. Results show that an insertion of 40 aa has little effect on the morphology and stability of the tubules. The protein follows the same sedimentation distribution of the native protein in a sucrose gradient. The 150 aa insertion does not affect the morphology of the tubules. However, the sedimentation profile differs from the native protein. A large insertion did not destabilize the tubule structure that may indicate a promising future for this antigen delivery system (de Lange, personal communication).
4.3.2.2 BTV and AHSV VP7 based vaccines

BTV and AHSV particulate vaccines have been based on VLPs and CLPs. Research showed that co-expression of VP2 and VP5 fail to form VLPs. However, VLPs are formed when VP2, VP5, VP7 and VP3 are co-expressed (French et al., 1990; Roy, 1996). The VLPs are highly immunogenic at lower doses providing long lasting protection against homologous BTV challenge (French et al., 1990; Roy, 1996). The problem with VLPs is the need for a multiple (quadruple) vector to co-express the proteins, as single or dual vectors to produce VLPs with different outer capsid protein amounts (Roy, 1996). VLPs containing AHSV VP2, VP5, VP7 and VP3 have also displayed long lasting protection in vaccinated horses (Roy et al., 1996). Co-expression of VP3 and VP7 of both BTV and AHSV form empty CLPs using a baculovirus expression system. The CLP does lack 5 VP7 trimers around each of the five-fold axis. VP2 and VP5 co-expression is required to incorporate these trimers (Hewat et al., 1994). These CLPs are just as infectious as intact VLPs in vector insects. However, the CLPs show a reduced infectivity in mammalian cells compared to intact virus (Wilson et al., 2000).

The BTV VP7 bottom domain comprises of tight helices that cannot be disturbed. Amino acids 168-170, A145 and G238 are accessible to the surface and located in the top domain (Roy et al., 1997). Insertions within 168-170 and A145 did not abrogate CLP formation while insertion within G238 did (Roy et al., 1997). A 15 aa insertion was also made in A145, generating chimeric CLPs with strong immunogenicity (Adler et al., 1998). Additions to the N terminus of 48 aa does not affect CLP formation when expressed in the presence of native VP7 to form mosaic CLPs. However a lower CLP yield was observed compared to C terminal additions (Le Blois and Roy, 1993; Roy, 1996; Tanaka et al., 1995).

AHSV VP7 has an increased immunogenicity as a purified product as VP7 is expressed at high levels producing the unique hexagonal disc-shaped crystals in baculovirus infected insect cells (Martínez-Torrecaudrada et al., 1996).

AHSV VP7 crystals inoculated into a mouse model showed promise as a subunit vaccine when challenged with heterologous AHSV-7 and may induce a protective T cell response (Wade-Evans et al., 1998). The mode of protection is assumed to be T cell related as passive antibody transfer from immunized mice failed to protect naïve mice from AHSV challenge (Burroughs et al., 1994; Wade-Evans et al., 1998). It has been shown that lower levels of protection are obtained using denatured VP7 crystals or bacterial GST-VP7 fusion protein when compared to VP7 crystals. The conformation of VP7 is thus important to elicit protection (Wade-Evans et al., 1997).
This prompted the Genetics Department of UP to investigate the use of AHSV VP7 as an antigen delivery system via the use of VLP, CLP or VP7 crystals. AHSV VP3 and VP7 co-infection in a baculovirus system self-assemble to form CLPs (Hewat et al., 1994). Dual expression vectors were constructed to express VP2, VP3 and VP5 and VP7 with VP7 and VP3 under control of the same promoter. This would ensure that particles are generated only in the presence of both dual vectors with no intermediate structures being formed. AHSV VP7 has a tendency to aggregate into large crystals even when co-expressed with VP3 that could then influence their incorporation into CLPs. This could account for the heterogeneous CLP assembly observed and the low yield. In AHSV VLPs, quantities of VP2 and VP5 on the outer layer varied. There was also a low VLP yield due to the suboptimal expression ratios of the proteins and the crystallization of VP7. The VLP strategy was abandoned in favour of a VP7 subunit vaccine (Maree, 2000).

Wade-Evans et al. (1998) showed that VP7 crystals were important for immunity. As the atomic structure of AHSV VP7 is similar to BTV VP7 several hydrophilic regions were chosen as insertion candidates based on the BTV VP7 crystallographic data. An insertion site was created in A177 (mu177) that occurs in the hydrophilic RGD loop. This resulted in an increase in hydrophilicity in that area with equal amounts of soluble and particulate structures observed on a sucrose gradient (Maree, 2000). An insertion in Q200 (mu200) resulted in a lesser increase in hydrophilicity as large areas of hydrophobic residues flanked the site. The mu200 protein was mostly recovered in the particulate fraction just as for unmodified VP7. Both unmodified VP7 and mu200 aggregated into crystals. Both mutants could assemble into CLPs when co-expressed with VP3. However, both unmodified VP7 and mu200 had a low CLP yield. Mu177 displayed an increased CLP yield compared to unmodified VP7. An increase in solubility of VP7 could thus lead to an increased yield in CLPs, as more soluble trimers would be available for assembly (Maree, 2000). This hypothesis was tested in unmodified VP7, mu200 and mu177. In order to increase CLP yield, L345 located in the bottom domain of VP7 was mutated to R in the unmodified VP7 as well as in mu177 and mu200. Mu177 showed no effect on solubility. The unmodified VP7 and mu200 showed a slight increase in solubility. Unfortunately no increase in CLP yield was found (Meyer, 2002). L345 was chosen as it was implicated into contributing to the insolubility of VP7 (Monastyrskaya et al., 1997).

A third insertion mutant was created at amino acid site 144 (mu144) within a hydrophilic region on VP7 by Riley (2003). The insertion mutant site did not abolish VP7 crystal formation. An increase in solubility was observed with increased trimers, aggregation into smaller and small particulate structures and decreased large particulate structures compared
to unmodified VP7. A 25 aa insertion of a neutralizing epitope on AHSV VP2 into site 144 did not affect the construct’s ability to form trimers. An increase in small particulate structures was present compared to VP7 and unmodified mu144 due to reduced hydrophobic interactions between the trimers with a reduced stability of the larger structures. The insertion influences trimer association into large particulate structures with the absence of the characteristic hexagonal shape of the observed crystals. The fractions containing the small particles were used in immunogenicity studies. A poor VP2 immune response was observed due to impurities in the fractions leading to dominant reactions against baculovirus and SF9 cell protein. The use of small particles lacking the hexagonal crystalline shape may also contribute to the poor immune response as it has been shown by Wade-Evans et al. (1998) that the crystal’s structure is important for the generation of an immune response (Riley, 2003).

Figure 1.2: A model of the AHSV VP7 trimer 3-D structure of the top domain using RASMOL software. The mutational inserts into the hydrophilic regions are indicated for aa site 144-145, 177-178, 200-201 in yellow, red and dark blue respectively (Maree, 2000).

Mu200 was employed to express a fusion product with another AHSV protein. The first 12 aa of AHSV-3 NS3 was inserted into site 200 in order to obtain NS3 antibodies. The insertion did not alter the protein and remained highly hydrophobic and insoluble. However, the chimera formed crystals with individual trimer layers (also called rosettes) visible due to the disruption of hydrophobic interactions between the sheets. VP7 monospecific polyclonal antibodies were raised with no detectable response against NS3. The poor NS3 specific immune response could be attributed to the hydrophobic NS3 insertion folding into the VP7
molecule. The peptide may also have been too small to be exposed on the surface of VP7 as site 200 is already less exposed on the surface compared to site 177 (Meiring, 2001).

The expression of foreign proteins within VP7 was continued using different epitopes and different VP7 mutant constructs. HIV-1 ELDKWA core epitope subtype B insertions into site 144 using a single repeat and site 177 using single and triple repeats were made. All of the constructs were significantly less soluble than the vector and assembled into the characteristic hexagonal crystals which were subsequently injected into mice. A humoral response against VP7 but no ELDKWA specific response was observed as was the case with the mu200 NS3 constructs. Again it is possible that the constructs were not well exposed on the surface with aggregation of crystals further shielding the epitope. A larger insert would guarantee exposure and assembly into particulate structures necessary for an immune reaction. Another solution would be to insert short epitopes with hydrophilic adaptors to increase the hydrophilicity, exposure and size of the insertion to ensure display (Meyer, 2002).

The possibility of using VP7 as a multiple epitope presentation system has also been explored. Multiple epitope competition would be lessened by distance, increasing the probability of an immune response against each epitope. Based on the double 144 and 200 site construct made by Riley (2003), which showed an increase in solubility but did not prevent crystal formation, an insertion site at position 177 was created. The VP7 144/177/200 construct showed an increase in aggregation with an increased amount of protein pelleted during sucrose gradient purification compared to unmodified VP7 (Van Rensburg, 2004). Storage methods as well as curbing the aggregation of this construct was investigated. Several storage methods were tested which did not alter the protein with freeze-drying causing the least VP7 144/177/200 aggregation. Sonication of VP7 144/177/200 yielded structures which varied in size while unmodified VP7 yielded distinct sized and density structures indicating instability of VP7 144/177/200. Sonication also lessened the aggregation of this vector. However, re-aggregation still needs to be curbed by the exploration of different non-polar solvents. VP7 144/177/200 crystals varied in size and shape with the most presenting as round plate like structures with a rough surface. High protein concentrations demonstrated web-like cables (Van Rensburg, 2004).

The effect of an epitope insertion in the new construct was investigated by using an AHSV VP2 19 aa insert in site 177 which resulted in increased aggregation and an increase in smaller particles. A 24 aa VP2 insert in site 144 resulted in decreased aggregation with an increase in smaller particles. A construct containing both the epitopes resembled the VP7
144/177/200 vector profile. All three constructs retained the structure of the vector. To test the size insertion limit of the vector, different sized hydrophilic VP2 insertions were made in site 177. A range of 100, 150, 200 and 250 aa inserts were tested. A highly hydrophilic insert decreased the level of aggregation. All four constructs resembled the vector structure. The results show the VP7 144/177/200 vector to be able to absorb the structural stress resulting from different hydrophilicity and sized inserts into site 177 (Van Rensburg, 2004). However, no information regarding the other sites as well as a triple insert and the sizes thereof in sites 144, 177 and 200 are available. Information is also lacking on immune responses generated by whole particulate vs. sonified protein and the use of non-polar solvents to curb aggregation of these highly aggregated proteins.

VP7 cell delivery using a viral delivery system was also explored. A bi-directional promoter of Lumpy skin disease virus (LSDV) has been identified and engineered for foreign gene expression. Early expression would induce CTL responses with late expression eliciting a humoral response (Fick, 1998). LSDV expressing the AHSV-9 VP7 protein formed VP7 crystals in infected Foetal bovine testis cells under control of the early promoter (Vos, 2001). To compare expression levels of early vs. late expression of VP7, VP7 was inserted into the engineered vector under control of the late promoter. The vector undergoes homologous recombination with the LSDV genome, inserting the promoter and foreign gene into an intergenic site. Multiple rounds of plaque purification were required to separate the recombinant from the native virus even though a mycophenolic acid and LacZ selection system was in place. The recombinant was eventually lost. Previous LSDV (type SA-Neethling, vaccine strain based) expression vector recombinants using the late promoter have also been unstable and lost. The LSDV delivery system was as such abandoned (Rutkowska, 2002).

5. Aims
The objectives of the research described in this dissertation form part of the evaluation of a novel vaccine delivery system based on the possibility of inserting foreign peptides into the AHSV VP7 protein and its assembly into particles. The system has potential for the development of recombinant vaccines against both human and animal pathogens. Several VP7 vectors have been developed for inserting peptides into amino acid sites 144, 177, 200 respectively as well as a triple vector for inserting peptides into these 3 sites simultaneously (VP7 144/177/200). Small epitopes and epitope repeats have been inserted into sites 144, 177 and 200. These previous studies were conducted with different epitopes such as a AHSV VP2 epitope into site 144, 12 aa of AHSV NS3 into site 200, HIV-1 subtype B ELDKWA core
epitope into site 144, site 177 and a triple ELDKWA epitope repeat into site 177. There has, however, not been a study focussed on comparing exactly the same insert in different mutant sites in VP7.

This study was focused on studying the effect of inserting different sized peptides into site 200 of the mutant VP7 vector. The structure, stability and ability to assemble into particles of these constructs were then compared to constructs in which the same peptides were inserted into site 177.

- The site 177 constructs containing HIV subtype C gp41 epitopes ALDSWK; RVAIERYLKD and dual (containing both HIV subtype C gp41 epitopes ALDSWK and RVAIERYLKD) all with flanking regions were previously prepared by Daria Rutkowska (Rutkowska, 2002). The insertion of identical RVLAIERYLKD and ALDSWK epitopes within site 200 will enable a direct comparison between sites 177 and site 200.

- By inserting peptides with relatively large flanking regions we should be able to determine the effect of peptide length on the stability and structure of the particles formed by the constructs.

- Previous HIV-1 subtype B ELDKWA core epitope insertions in VP7 sites 144 and 177 failed to generate a specific ELDKWA response in mice. A triple ELDKWA epitope repeat in site 177 also failed to generate a response (Meyer, 2002). The inclusion of the flanking regions should increase the antigenicity of these HIV epitopes.

- The aims were twofold, generation of analogous mutant 200 recombinants to mutant 177 recombinants already constructed and secondly comparison of the two vector sites.

Strategy:

2. Expression of the chimeric VP7 mutants using the Baculovirus expression system.
3. Determine the structural effects these insertions in site 200 of VP7 with density sedimentation analysis and Scanning Electron Microscopy.

4. To compare the effect of the insertions in site 200 to that of site 177 of VP7 with density sedimentation analysis and Scanning Electron Microscopy.

5. Comparison of the antigenicity of the HIV epitopes in site 177 and 200 of VP7 via Western blot.
CHAPTER 2

THE EFFECTS OF HIV-1 SUBTYPE C EPITOPES ON AHSV VP7 MUTANT SITE 200 AND COMPARISON WITH VP7 MUTANT SITE 177.

2.1 Introduction

The core capsid protein VP7 of AHSV is being explored as a possible particulate vaccine delivery system for the display of small peptides to the immune system. The suitability of VP7 for this type of vaccine is based on the observation that AHSV VP7 aggregates into flat hexagonal crystalline particles. This tendency to aggregate is due to the hydrophobicity of the VP7 molecule (Basak et al., 1996). VP7 crystals that were used as a subunit vaccine in a mouse model were shown to be effective against challenge with heterologous AHSV-7 due to possible T cell activation, as no neutralizing antibodies were observed (Wade-Evans et al., 1998).

As a step towards investigating the use of AHSV VP7 as a particulate vaccine a number of different AHSV-9 VP7 mutants were generated to serve as epitope display vectors. Crystallographic data of BTV VP7 which has a similar atomic structure to AHSV VP7 was used to identify hydrophilic regions in the top domain which could be used as insertion sites. Site 177 was created within the hydrophilic RGD loop. Insertion sites 144 and 200 were created in hydrophilic regions identified on VP7. Amino acid position 144, 177, and 200 were modified by introducing restriction enzyme sites at the respective amino acid position by PCR based methodology (Maree, 2000; Riley, 2003). These three mutant vectors now need to be assessed and compared with regard to the size of epitopes, the number of insertions that can be inserted simultaneously, and the effect these have on expression, particle assembly as well as the conformation and stability of the modified VP7 vector. Ultimately the immunogenicity of these constructs also need to be evaluated. Various epitopes have previously been inserted into sites 200 and 177. The insertion of the first 12 aa of AHSV-3 NS3 in site 200 and HIV-1 ELDKWA core epitope insertion into site 177 did not incorporate flanking regions. Both failed to show an insert specific immune response (Meiring, 2001; Meyer, 2002). The incorporation of flanking regions to facilitate the correct conformation of the insert could produce an immune response. Epitopes with flanking regions have thus far not been investigated in the VP7 vaccine delivery system. The inclusion of flanking regions also explores the size range that can be inserted within the site.
The structure, particle assembly and stability can be assessed using a variety of different peptides such as HIV-1 subtype C epitopes already cloned into mutant 177, (Rutkowska, 2002), which will be useful in comparing the effects of the same peptides in mutant 200. This will yield information on differences that may be present with the different vectors. There are three mutant 177 constructs. The first contains the DNA fragment that encodes the RVLAIERYLKD epitope and its flanking regions. The second contains the DNA fragment that encodes the ALDSWK epitope and its flanking regions. The dual construct contains both the aforementioned DNA fragments. The dual construct containing both the DNA fragments were chosen as it uses exactly the same fragments as in the single constructs. The difference in effect that the different inserts have on the VP7 particle will be comparable. It also adds a larger insert to the VP7 which would yield information on the maximum size of the insert that could still form VP7 particles.

The ALDSWK and RVLAIERYLKD epitopes were chosen as they are relevant to South Africa and other African countries dealing with the spread of HIV-1 subtype C. These immunodominant gp41 epitopes are found in the N-terminal region and includes the cytotoxic T lymphocyte epitope (591-602 aa; AVERYLKDQQLL) and the ectodomain (671-676 aa; ALDKWA) which is recognized by the neutralizing mAb 2F5 (Downing et al., 2000). The mAb 2F5 has been shown to neutralize several HIV-1 subtypes from Africa, Asia, America and Europe. HIV-1 subtype C is sensitive to mAb 2F5 as it binds to the gp41 complex involved in viral fusion (Morris et al., 2000; Lu et al., 2000; Brown et al., 1995).

Once the epitopes and their flanking regions have been obtained via PCR, the DNA fragments will be cloned into a suitable plasmid vector which is pFastbac1 containing the VP7 mutant 200 gene. The baculovirus expression system will then be used to express the VP7 fusion proteins. This system was chosen for having high protein expression levels, with no size limitations on the insert. Expression of multiple genes can occur simultaneously and is safe as the baculovirus has a narrow host range which is restricted to insects. Posttranslational modifications occur within the insect cells which results in the protein exhibiting characteristics of their native counterparts (Massotte et al., 2003). The Bac-to-Bac™ expression system uses Tn7 mediated transposition in E. coli for recombinant bacmid production. The bacmid is a recombinant baculovirus genome (based on the Autographica californica nuclear polyhedrosis virus, AcNPV genome) that contains a mini-F replicon in the polyhedron locus, a kanamycin resistance gene and a mini att-Tn7 (target site for bacterial transposon Tn7) within the LacZα complementation region. The bacmid can replicate as a plasmid in E. coli and infect Sf9 cells (Leusch et al., 1995).
A donor plasmid is needed which contains the polyhedron promoter flanked on either side by Tn7, a SV40 poly (A) signal and a multiple cloning site. The ATG of the polyhedron has been mutated to ATT, thus requiring the foreign gene to contain its own ATG and ORF for expression. The donor plasmid is used to transform *E. coli* DH10 BAC cells that contain the bacmid and the donor plasmid. This causes a transposition event thereby inserting the foreign gene and polyhedron promoter into the bacmid genome. The recombinant bacmid DNA is used to transfect insect cells, which produces baculovirus expressing the foreign gene (Leusch et al., 1995).

The effect of the various insertions on the VP7 particle will be investigated by sedimentation analysis using density gradient centrifugation and Scanning Electron Microscopy. This will answer questions on whether the insert changes the VP7 particle in density or shape. Density gradient centrifugation can be divided into rate zonal and isopycnic conditions. During rate zonal centrifugation particles sediment through the gradient into separate zones based on the size of the particles. Zonal centrifugation conditions only apply as long as the density of the particle is higher than the density of the gradient medium at that specific position within that gradient (Griffith, 1976).

During isopycnic density gradient centrifugation the particle sediments to that position in the gradient where the particle density equals that of the density of the gradient medium (Turner et al., 1997). When differently sized particles are involved on a sucrose gradient, density analysis will involve both zonal and isopycnic centrifugation conditions with the large particles reaching isopycnic conditions before the smaller particles (Cooper, 2000). Experiments can therefore contain both rate zonal and isopycnic centrifugation. The gradient will be of such a density range that one component sediments at the bottom of the tube (pellet) and another sediments at its isopycnic position (Griffith, 1976). A combination of rate zonal and isopycnic centrifugation achieved by short centrifugation conditions such as 90 minutes could yield information on both particle size and density.

The effect of the HIV inserts on VP7 particles are unknown and therefore their sucrose density gradient profiles cannot be predicted. Chimeric particles may vary in size compared to VP7 mutant 200 particles. The chimeric particles may also vary in density. It is also possible that particle formation may be abolished. It is possible that the particles are associated with membrane or lipid cell material that will reduce the density of the particles. If particles are formed the effect of the epitopes on the hexagonal VP7 crystal can be visualized using Scanning Electron Microscopy.
We do not know if the VP7 environment will affect the antigenicity of the epitopes. Western blotting is effective in testing the antigenicity of a protein before investing in costly animal trials.

A western blot with HIV serum will detect if the epitopes are recognized as previous research within the Genetics department (as discussed previously) failed to show insert specific immune responses using VP7mu200, VP7mu177 and VP7mu144 display vectors. We hypothesize that with the increased flanking regions a conformationally correct epitope will be displayed which will be recognized by a HIV serum.

The antigenicity of the inserts and the effects of the three insertions namely only RVLAIERYLKD or ALDSWK and both RVLAIERYLKD and ALDSWK HIV-1 subtype C epitopes with their flanking regions on mutant 200 expression, particle assembly, stability and structure were investigated and compared with information from site 177 with identical inserts.
2.2 Materials and Methods

2.2.1 Materials

**Plasmids:** pFb7mu200 was provided by T. Meiring and constructed by F. Maree, Department of Genetics, UP. HIV-1 subtype C, DU151 containing gp160 (that contains gp120 and gp41) in pFastbac1 was obtained from Dr Lynn Morris, (NICD).

**Chemicals and biological products:** Restriction endonucleases (Boehringer Mannheim/Roche), Molecular weight markers (Promega), Glassmilk (Bio 101), New Wash (Bio 101), Alkaline phosphatase and Buffer (Promega), High Pure PCR product purification Kit (Roche). Geno Pure Plasmid Midi Kit (Roche), ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit v.3 (Perkin Elmer Biosystems), Cellfectin™ (Invitrogen), DH10Bac cells (Life technologies), Grace’s insect cell medium (Highveld Biological Ltd), Foetal Bovine Serum (Highveld Biological Ltd), Penicillin and Streptomycin antibiotic solution (Roche), Neutral Red (Sigma). HIV-1 subtype C antiserum obtained from infected individuals from Dr M. Papaphanasopolous, (NICD).

2.2.2 Plasmid DNA extraction

The alkaline lysis method (Sambrook et al., 1989) was used to obtain plasmid DNA in small and large scale preparations. Briefly, bacterial cells were collected from overnight cultures. Pellets were resuspended in solution I (50 mM glucose; 10 mM EDTA; 25 mM Tris, pH 8.0). Lysis was achieved by adding 0.2 M NaOH, 1 % SDS to the samples. 3 M sodium acetate pH 4.8 was also added after which cell debris; genomic DNA and high molecular weight RNA were removed by centrifugation. Plasmid DNA was recovered by 96 % ethanol precipitation and centrifugation. Excess salt was removed with a 70 % ethanol wash. Pellets were dried under vacuum and resuspended in UHQ. In some cases Nucleobond AX-100 cartridges were used to further purify plasmid using the high copy number plasmid procedure of the Geno Pure Plasmid Midi kit (Roche). Isolations were done according to the manufacturer’s recommendations. The lysate obtained was cleared by centrifugation at 15 000 rpm for 45 min, deviating from the original protocol. These highly purified products were used in sequencing reactions.

2.2.3 Phenol-chloroform purification

Small scale plasmid samples were purified by removal of protein by phenol-chloroform extraction. 1 x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added to a final volume of 400 µl. An equal volume of a 25: 24: 1 solution of phenol : chloroform : isoamyl alcohol was added followed by centrifugation at 13 000 rpm for 5 min. The upper aqueous phase was removed and extracted twice using the same centrifugation conditions with one volume of chloroform. Sodium acetate pH 7 was added to the upper aqueous phase and 96 % ethanol and incubated at -20°C for 1 hr followed by the ethanol precipitation and wash step described above.

2.2.4 Restriction Endonuclease digestion

Restriction enzymes with their specified buffers were used according to manufacturer’s recommendations. Approximately 2 µg of column purified DNA or column purified PCR product was digested with the appropriate restriction enzyme at 37°C for 5 hrs. After the first enzyme digestion, DNA was recovered by ethanol precipitation and collected by centrifugation. Pellets were dried under vacuum and resuspended in UHQ for digestion with other restriction enzymes for 5 hrs.
2.2.5 PCR

Primers as outlined in Table 1 were designed to amplify specific HIV epitope regions on the HIV-1 gp160 plasmid clone. The VP7 specific and pFastbac primers that were used for sequencing and PCR analysis of recombinant constructs are also described.

**Table 1: Primers used for construction and evaluation of modified AHSV VP7 mutant 200 inserts.**

<table>
<thead>
<tr>
<th>Primer Abbreviation</th>
<th>Primer Sequence</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>RilaFWXS abbreviated to R1</td>
<td>5’CCCTCTAGAGCTATACAGGCGCAACAG 3’&lt;sup&gt;XbaI&lt;/sup&gt;</td>
<td>1615 bp - 1632 bp on the gp160 HIV-1 subtype C, DU 151 sequence (NCBI Genbank accession number AY043173.1).</td>
</tr>
<tr>
<td>RilaRV abbreviated to R2</td>
<td>5’CCCGTCGACGAATTCCACAGCAGTGTCAGAT 3’&lt;sup&gt;EcoRI SalI&lt;/sup&gt;</td>
<td>Binds at 1752 bp - 1769 bp on the gp160 sequence.</td>
</tr>
<tr>
<td>AldFWXS abbreviated to A1</td>
<td>5’CCCTCTAGAGAATTCAGGTTACTTGAGACTCG 3’&lt;sup&gt;XbaI EcoRI&lt;/sup&gt;</td>
<td>Binds at 1873 bp - 1890 bp on the gp160 sequence.</td>
</tr>
<tr>
<td>AldRV abbreviated to A2</td>
<td>5’CCCGTCGACGCCTCCTACTATCATGAT 3’&lt;sup&gt;SalI&lt;/sup&gt;</td>
<td>Binds at 2001 bp - 2018 bp on the gp160 sequence.</td>
</tr>
<tr>
<td>DrFW abbreviated to D1</td>
<td>5’TACGTACCGCAAGGTCG 3’</td>
<td>Binds at 439 bp - 456 bp on the AHSV serotype 9 VP7 sequence (NCBI Genbank accession number U90337).</td>
</tr>
<tr>
<td>HkRV abbreviated to H2</td>
<td>5’GAACCGTGTCTAGCGATC 3’</td>
<td>Binds at 780 bp - 797 bp on the AHSV serotype 9 VP7 sequence.</td>
</tr>
<tr>
<td>pPol(h)FW abbreviated to P1</td>
<td>5’TTCCGGATTATTGATTCCAC 3’</td>
<td>Binds at 3996 bp - 4013 bp on pFastbac1 in the multiple cloning site region.</td>
</tr>
<tr>
<td>pFastbac1RV abbreviated to P2</td>
<td>5’GTATGGCTGATTGATCCTC 3’</td>
<td>Binds at the 3’ end of the multiple cloning site within pFastbac1 at 4155 bp -4175 bp.</td>
</tr>
</tbody>
</table>

The PCR conditions and expected product sizes of the different primer combinations used are described in Table 2.
Table 2: PCR conditions.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Product size</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 + R2</td>
<td>176 bp</td>
<td>94°C, 2 min; repeat the following for 30 cycles: [94°C, 45 sec; 60°C, 45 sec; 72°C, 45 sec]; followed by 72°C, 10 min.</td>
</tr>
<tr>
<td>A1 + A2</td>
<td>167 bp</td>
<td></td>
</tr>
<tr>
<td>D1 + H2</td>
<td>341 bp (VP7 without an insert)</td>
<td></td>
</tr>
<tr>
<td>P1 + P2</td>
<td>1344 bp (with VP7 and no insert)</td>
<td>93°C, 3 min; repeat the following for 35 cycles: [94°C, 45 sec; 55.5°C, 45 sec; 72°C, 10 min]; followed by 72°C, 5 min.</td>
</tr>
<tr>
<td>P1 + H2</td>
<td>815 bp (with VP7 and no insert)</td>
<td></td>
</tr>
</tbody>
</table>

PCR products were analyzed on 1-2 % agarose gels. Where necessary, the PCR products were purified using the PCR Product Purification kit (Roche) according to the manufacturer’s specifications for further use in sequencing or restriction enzyme digestion.

2.2.6 Dephosphorylation
The vector was dephosphorylated using 0.5 U alkaline phosphatase (Promega) and 2 U of 10 x dephosphorylation buffer (0.5 M Tris-HCl, 1 mM EDTA, pH 8.5) with incubation at 37°C for 20 min according to the manufacturer’s recommendations. Dephosphorylated products were purified by phenol-chloroform extraction and ethanol precipitation (Sambrook et al., 1989).

2.2.7 Ligation
Sticky-end ligation was performed at 16°C overnight in the presence of 1 unit T4 DNA ligase and 2 units 1x ligation Buffer (660 mM Tris-HCL; 10 mM DTT; 50 mM MgCl2; 10 mM ATP, pH 7.5) with an insert: vector molar ratio of 7:1.

2.2.8 Preparation of competent *E. coli* XL1-BLUES and transformation
Competent cells were prepared using the CaCl2 method (Sambrook et al., 1989). An overnight culture was used to inoculate a volume of LB Broth (bacto-tryptone; bacto-yeast extract; NaCl pH 7.4) supplemented with 12.5 µg / ml Tetracycline and allowed to grow at 37°C with shaking until log phase growth (OD<sub>550</sub> = 0.45-0.5) was achieved. Cells were harvested by centrifugation and resuspended in ice cold 50 mM CaCl<sub>2</sub> followed by incubation on ice. Cells were pelleted by centrifugation and resuspended in 1/20 50 mM CaCl<sub>2</sub>. Cells were incubated on ice for an hour before use.

Transformation was induced by heat shock (Sambrook et al., 1989). The ligation mix and competent cells were incubated on ice for 30 min. A heat shock at 42°C was performed for 90 sec, followed by incubation on ice for 2 min. LB Broth was added to the cultures and incubated at 37°C for 1 hr with shaking. Cultures were plated out on 100 µg / ml Ampicillin and 12.5 µg / ml Tetracycline LB-agar plates and incubated overnight at 37°C.

2.2.9 Sequencing
DNA templates were purified using the Geno Pure Plasmid Midi kit or PCR product purification kit (Roche) according to the manufacturer’s recommendations. DNA concentrations were calculated using absorbency readings from a spectrophotometer at 260 nm with a DNA extinction coefficient of 1 A<sub>260</sub> = 50 µg / µl. Sequencing reactions were performed using the ABI PRISM™ Big Dye Terminator Cycle sequencing Ready Reaction kit v.03 (Perkin Elmer Biosystems) in a GeneAmp 9600 ABI thermal cycler. Templates of between 90 ng (± 300 bp PCR product), 250 ng (± 1200 bp PCR product) and ± 500 ng (plasmid DNA) were used in full reactions with a primer concentration of 3.2 pmol / µl. The PCR conditions used were: 96°C 10 sec; 50°C 45 sec; 60°C 4 min, repeated for 25 cycles.
Unincorporated dye terminators were removed by ethanol precipitation. A volume of 2 µl 3 M sodium acetate pH 4.8 and 50 µl 99.9 % ethanol (at RT) were added to the reaction and left on ice for 10 min. DNA was pelleted using a desktop centrifuge for 30 min. A 70% ethanol (at RT) wash was performed for 20 min in a desktop centrifuge. The pellet was vacuum dried. The reactions were analyzed using an ABI PRISM 377 / 3100 sequencer. Dried samples were resuspended in 3 µl sequencing loading buffer (5:1 deionised formamide: 25 mM EDTA, pH 8 containing 50 mg / ml dextran blue) before loading. The samples were denatured at 95°C for 2 min. 1.5 µl of the sample was loaded onto a 4 % denaturing polyacrylamide gel and electrophoresed for 7 hrs at 1.6 kV. Sequences were analyzed using the ABI PRISM Sequencing Analysis™ program and the ABI PRISM Sequence Navigator™ program.

2.2.10 Cells and media
*Spodoptera frugiperda* (SF9) cells were used for baculovirus expression of proteins. Cells were grown in suspension culture at 27°C in Grace’s insect medium supplemented with 10 % Fetal calf serum and antibiotics (Penicillin, Streptomycin and Fungizone).

2.2.11 Transposition in *E.coli* DH10Bac cells
Competent DH10Bac cells were made using the PEG / DMSO method as described by Chung and Miller, (1988). Briefly, overnight cultures were used to inoculate a volume of LB Broth (Kanamycin and Tetracyclin supplemented) and grown to early log phase. Cells were pelleted and resuspended in 1/10 volume of ice cold TSB (LB Broth; 10 % (w / v) PEG; 5 % (v / v) DMSO; 10 mM MgCl₂; 10 mM MgSO₄). Cells were kept on ice for 20 min. A volume of 200 µl of the competent cells and ± 0.5 - 1 µg of plasmid DNA were incubated on ice for 30 min. A 45 sec heat shock at 42°C was applied and then left on ice for 2 min. 900 µl TSBG (TSB + 20mM glucose) was added and incubated at 37°C with shaking for 4 hrs. Aliquots were plated out onto Kanamycin (50 µg / ml), Gentamycin (7 µg / ml), Tetracycline (10 µl /ml), X-Gal (300 µg / ml) and IPTG (40 µg / ml) LB-agar plates. Plates were incubated for 48 hrs at 37°C. White colonies (recombinants) were replica plated to confirm their identity. White colonies were used to inoculate mini cultures overnight. The Bacmid DNA was isolated using a modified alkaline lysis method for large DNA molecules.

2.2.12 Recombinant bacmid DNA isolation
A protocol developed for isolation of large plasmids (>100 kb) and adapted for high molecular weight bacmid DNA isolation was followed from the Baculovirus Expression Sytem Manual (Life technologies). Cell pellets obtained from 2 ml overnight cultures (supplemented with 50 µg / ml Kanamycin, 7 µg / ml Gentamycin, 10 µg / ml Tetracycline) were resuspended in 300 µl solution I (15 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µg / ml RNaseA ) followed by addition of 300 µl solution II (0.2 N NaOH; 1 % SDS ). Tubes were gently inverted and incubated at RT for 5 min. A volume of 300 µl 3 M potassium acetate pH 5.5 was slowly added and placed on ice for 5 - 10 min. The supernatant was collected after centrifugation at maximum speed for 10 min and added to 800 µl absolute isopropanol (RT). Samples were incubated overnight at -20°C. DNA was pelleted by centrifugation and washed with 70 % ethanol. Pellets were air dried in a laminar flow hood and resuspended in 25 µl UHQ. The presence of DNA was confirmed on an agarose gel.

2.2.13 Screening of recombinant bacmid DNA
Recombinant bacmid DNA samples were selected by means of PCR analysis. The 25 ng template was added to a 50 µl reaction (7.5 mM dNTP mix; 75 mM MgCl₂) containing the P1 and H2 primers. Primers and PCR conditions are described in section 2.2.5 Table 1 and 2. Samples were visualized on an agarose gel.

2.2.14 Sf9 cell transfection with bacmid DNA
Cells were seeded in 35 mm six well tissue culture plates (Nunclon™) at 1 x 10⁶ cells per well. Cells were allowed to attach for at least 1 hr at RT. Transfection mixtures were
prepared as follows: 6 µl of recombinant bacmid DNA was diluted into 100 µl Grace’s insect medium without supplementation with serum or antibiotics). Solution II contained 6 µl Cellfectin™ (GIBCO BRL) diluted in 100 µl unsupplemented Grace’s insect medium. Solution II was added to solution I and mixed by inversion. The solution was incubated at RT for 45 min. The cells were washed once with unsupplemented Grace’s insect medium. A volume of 800 µl unsupplemented Grace’s insect medium was added to the lipid-DNA complexes and mixed gently. The cells were overlaid with the solution containing the lipid-DNA complexes and incubated in a Tupperware™ container for 5 hrs in a 27°C tissue culture incubator. Transfection mixtures were then replaced with 2 ml Grace’s containing serum and appropriate antibiotics. The cells were incubated for 72 hrs in a 27°C incubator after which the viral supernatant was harvested and stored at 4°C.

2.2.15 Harvesting of whole cell suspensions for protein analysis
Cells were seeded in a 24 well tissue culture plate (Nunclon™) at 0.6 x 10^6 cells / well. Cells were allowed to attach for 1 hr after which the media was replaced with viral supernatant and Grace’s insect medium. SF9 cells were either mock-infected or infected with the wild type baculovirus or recombinant viruses at ± 5 MOI (pfu / ml). Cells were incubated at 27°C for 96 hrs. Cells were harvested and washed by means of 1 x PBS (137 mM NaCl; 2.6 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4 pH 7.4) resuspension and centrifugation.

2.2.16 SDS polyacrylamide gel electrophoresis
Samples containing an equal volume of 2 x protein solvent buffer (125 mM Tris-HCl pH 6.8; 4 % SDS; 20 % glycerol; 10 % 2-mercaptoethanol; 0.002 % bromophenol blue) were loaded onto discontinuous 10-12 % SDS-PAGE gels as described by Laemmli, (1970). Briefly 10 or 12 % separating gels (0.375 M Tris-HCl pH 8.8; 0.1 % SDS) with 5 % stacking gels (0.125 M Tris-HCl pH 6.8; 0.1 % SDS) were prepared from a 30 % acrylamide; 0.8 % bisacrylamide stock. Polymerization of gels occurred by addition of 0.008 % (v / v) tetra-methyl-ethylene-diamine (TEMED) and 0.08 % (w / v) ammonium peroxysulfate. Denatured samples were loaded after boiling for 10 min. Samples were analysed with either 16 x 18 cm Hoefer Sturdier Slab gel electrophoresis units or 7 x 10 cm Hoefer Mighty Small™II SE250 electrophoresis units in 1 x TGS (0.025 M Tris-HCl pH 8.3; 0.192 M glycine; 0.1 % SDS).

2.2.17 Coomassie Brilliant Blue staining
Gels were stained in 0.125 % coomassie brilliant blue; 50 % methanol solution for 20 min at RT. Gels were destained in 5 % acetic acid; 5 % methanol.

2.2.18 Plaque titrations and amplification of viral stocks
A number of 75 cm^3 flask Sf9 cell monolayers were infected with 0.01 MOI (pfu / cell) of the recombinant viral stocks in 5 ml medium and incubated at RT for 1 hr. Medium was added to a final volume of 14 ml. After 72 hr incubation at 27°C the supernatants were collected and filter sterilized (0.22 µm Millipore filter). Viral stock aliquots were frozen at -70°C and working stocks kept at 4°C. A six well plate was seeded with 1.2 x 10^6 cells / well and allowed to attach at RT for 1 hr to determine the viral titer. A dilution series ranging from 10^-1 - 10^-9 was made. The medium was replaced with the viral dilutions and left for 2 hrs at RT. The dilutions were replaced with 2 ml of a 1 % agarose / Grace’s insect medium mixture in equal volumes at 37°C. Cells were incubated at 27°C for 4 days. One ml neutral red (1 mg / ml in ddH_2O diluted 10 x with Grace’s insect medium) was added to the wells and incubated at 27°C for 5 hrs. The neutral red solution was removed and plates were incubated overnight at 27°C. Plaques were visible as light red patches against a dark red background. The viral titer was determined from the number of plaques visible in a dilution.

2.2.19 Large scale protein expression
Sf9 monolayers in 75 cm^3 culture flasks (1 x 10^7 cells / ml) were infected with the recombinant baculoviruses at a MOI of 5-10 pfu / cell and incubated at 27°C. Cells were harvested at 96 hpi. Cells were harvested from the supernatant and the monolayer by low
speed centrifugation (3,000 rpm, 15 min). Cells were resuspended in 1 ml TNN lysis buffer (50 mM Tris-HCl pH 8.0; 150 mM NaCl; 0.5 % Nonidet P-40) and left on ice for 30 min.

2.2.20 Sucrose density gradient purification and ultracentrifugation
The cell lysates were loaded onto a 50 - 70 % (w / v) discontinuous sucrose gradient in 1 x PBS. The sucrose gradients were centrifuged at 12,000 rpm for 1 hr 15 min at 4°C in a SW 50.1 Beckman rotor. Equal volumes of fractions were collected and analyzed by SDS-PAGE. Samples of fractions were diluted 1 / 6 with 1 x PBS. The particles were sedimented by centrifugation at 5,000 rpm for 45 min to remove sucrose before loading onto SDS-PAGE gels. Fractions containing the protein of interest were also diluted 1 / 6 in 1 x PBS and the particles collected by centrifugation at 5,000 rpm for 45 min for Electron Microscope analysis.

2.2.21 Mechanical lysis and ultracentrifugation
SF9 monolayers in 75 cm³ culture flasks (1 x 10⁷ cells / ml) were infected with the recombinant baculoviruses at a MOI of 5-10 pfu / cell and incubated at 27°C. Cells were harvested at 96 hpi as described in section 2.2.19. The cells were not chemically lysed with TNN lysis buffer. The cells were resuspended in 1 ml 1 x PBS and mechanically lysed with a dounce homogenizer using 15 strokes. The cell lysates were loaded onto a 50 - 70 % (w / v) discontinuous sucrose gradient in 1 x PBS and subjected to ultracentrifugation as described in section 2.2.20. Fractions were collected as described above and diluted 1 / 6 in TNN lysis buffer. The protein was collected after centrifugation at 5,000 rpm for 45 min.

2.2.22 Hydrophilicity analysis
Hydrophilicity plot predictions were made using the Hopp and Woods predictive model (Hopp & Woods, 1981; 1983) from the ANTHEPROT software (Geourjon et al., 1991; Geourjon & Deleage, 1995).

2.2.23 Scanning Electron Microscopy
Sucrose gradient purified proteins were fixed with 2.5 % gluteraldehyde in 0.075 M KH₂PO₄ / Na₂HPO₄ (Na-K-P) buffer pH 7.4 for 10 min. This solution containing the protein was filtered onto a 0.22 µm nylon filter, washed six times with a 1 : 1 ddH₂O and Na-K-P buffer, waiting 10 min between washes and dehydrated by successive treatments with 50 %, 70 %, 90 % and 6 times with 100 % ethanol for 10 min each. The filters were dried in a critical point drier with liquid CO₂ and mounted onto a stub before being spatter coated with gold. The samples were viewed in a JEOL 840 Scanning Electron Microscope at 5 kV.

2.2.24 Western blot
Proteins were separated on a 12 % polyacrylamide gel for 90 min at 140 V and blotted onto a nitrocellulose membrane (Hybond C) for 90 min. Membranes were washed in Wash Buffer (0.05 % Tween 20 in 1 x PBS pH 7.4) for 5 min. The membranes were blocked with 1 % skimmed milk powder in 1 x PBS pH 7.4 for 30 min at RT to prevent non-specific binding. Membranes were incubated overnight with 1 / 200 primary antibody while shaking. The membranes were washed three times in washing buffer for 5 min. The 1 / 250 secondary antibody (horseradish peroxidase conjugated anti-rabbit IgG) was incubated with the membrane for 1 hr while shaking at RT. Membranes were washed 3 times in wash buffer for 5 min. The membrane was incubated in 1 x PBS containing the peroxidase enzyme substrate (4-chloro-1-naphtol in 20 ml ice cold methanol) and H₂O₂. The colour reaction was allowed to develop in the dark, after which the blot was rinsed and dried.
2.3 Results

2.3.1 Construction of AHSV VP7 mutant 200 recombinants containing HIV-1 subtype C epitopes

The plasmid vector pFb7mu200, constructed by Francois Maree (2000), was used for cloning the HIV epitopes. It consists of a pFastbac1 backbone into which a modified version of the AHSV VP7 gene, pFb7mu200, was cloned (Maree, 2000). The VP7 modification involved the insertion of an 18 bp DNA fragment that include 3 restriction enzyme sites (HindIII, XbaI and SalI) into the VP7 region that encodes the top domain. This insertion makes it possible to clone a DNA fragment that will be expressed as a VP7 fusion protein between amino acid 200 and 201. The inserted 18 bp region encode 6 new amino acids, KLSRVD.

The sequence of HIV-1 subtype C, DU151 and a sample of the gp160 gene cloned into pFastbac1 was provided by Sibusiso Nkosi (NICD, previously known as NIV). The sequence was used to design primers for amplifying the regions of interest on the HIV-1 subtype C gp160 gene. The DNA fragments that encode the HIV epitope inserts were obtained from a HIV-1 gp160 clone using a PCR strategy. The primer sets included restriction enzyme tags for cloning the fragments into the XbaI and SalI sites created within VP7. A unique restriction enzyme, EcoRI, was included in the primer design to facilitate construction of a dual recombinant containing both HIV epitope inserts. In order to increase restriction enzyme efficiency, 3 cytosine bases were incorporated immediately after the restriction enzyme sites. The primers and PCR conditions have been described in section 2.2.5 Tables 1 and 2.

Primers were designed to amplify a 171 bp DNA fragment that encodes the RVLAIERYLKD (Rila) epitope and its flanking regions. The R1 primer contained an XbaI restriction enzyme site at the 5’ end. The R2 primer contained EcoRI and SalI sites at the 3’ end (schematically shown in Fig. 2.1). This design facilitates the cloning of another insert behind the first using the EcoRI and SalI sites. The R1 and R2 primers in combination will amplify the 171 bp sequence that encodes the Rila epitope and its flanking regions. A short description including the chosen nomenclature for the proposed inserts and future constructs are presented in Table 3. A Rila PCR product in the expected size range of 171 bp (Fig. 2.2 lane 1) was obtained. Insertion of the 171 bp DNA fragment will add a total of 53 amino acids to the VP7 protein.
Figure 2.1: Schematic representation of the construction of the Rila and Ald inserts that were cloned into pFb7mu200 to obtain the pFb7mu200-R and pFb7mu200-A recombinants.
Primers were also designed to amplify a 162 bp DNA fragment that encodes the ALDSWK (Ald) epitope and its flanking regions. The 5’ end of the A1 primer contained XbaI and EcoRI restriction enzyme sites. The 3’ end of the A2 primer contained the SalI site (as shown in Fig. 2.1). The restriction enzyme tagged ends of the PCR products allowed for directional cloning into suitably digested pFb7mu200 using the XbaI and SalI sites. Amplification of the Ald epitope with its flanking regions by means of the A1 and A2 primers should yield a 162 bp product. Insertion of this DNA fragment in site 200 will add 50 amino acids to the VP7 protein. Ald amplification products were obtained in the expected size range of 162 bp (Fig. 2.2 lane 2). Once the inserts were obtained it was possible to generate the recombinants of the pFb7mu200 vector.

**Table 3: Construct description.**

<table>
<thead>
<tr>
<th>Description</th>
<th>Epitope</th>
<th>Primers used to amplify DNA fragments for cloning</th>
<th>DNA fragment</th>
<th>VP7 Construct</th>
<th>Fusion protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>R Single recombinant</td>
<td>RVLAIELYLDK</td>
<td>R1+ R2 (refer to Table 1 for target sequence)</td>
<td>Rila (171 bp) 33 bp core epitope flanked by 120 bp as well as the XbaI, SalI and EcoRI restriction enzyme sites</td>
<td>pFb7mu200-R</td>
<td>VP7mu200-R</td>
</tr>
<tr>
<td>A Single recombinant</td>
<td>ALDSWK</td>
<td>A1+ A2 (refer to Table 1 for target sequence)</td>
<td>Ald (162 bp) 18 bp core epitope flanked by 126 bp as well as the XbaI, SalI and EcoRI restriction enzyme sites</td>
<td>pFb7mu200-A</td>
<td>VP7mu200-A</td>
</tr>
<tr>
<td>RA Dual recombinant</td>
<td>RVLAIELYLDK and ALDSWK</td>
<td>A1+ A2 for Ald DNA fragment</td>
<td>Rila and Ald fragment as above ligated together via the EcoRI restriction enzyme site</td>
<td>pFb7mu200-RA</td>
<td>VP7mu200-RA</td>
</tr>
</tbody>
</table>
Figure 2.2: 1% Agarose gel electrophoresis of the PCR products obtained after amplification of the HIV-1 subtype C gp160 gene with the R1/R2 and A1/A2 primers (Table 1).

Lane 1: DNA molecular weight marker ΦX174.
Lane 2: Rila PCR product.
Lane 3: Ald PCR product.
The vector and PCR products were prepared for cloning by restriction enzyme digestion of XbaI and SalI sites as described in section 2.2.4. The cloning procedure is schematically shown in Fig. 2.3. The two restriction enzyme digestions were carried out separately to ensure complete digestion of the vector and inserts. The vector was dephosphorylated to prevent religation and the ligation was carried out as described in section 2.2.7 with a 1:7 molar ratio of vector to insert increasing the probability of obtaining successful ligations. XL1-Blue cells were transformed according to the protocol in section 2.2.8. The transformation reactions were plated out onto Tet + Amp + agar plates and incubated overnight at 37°C. Plasmid DNA was isolated from selected colonies using the protocol in section 2.2.2.

### 2.3.2 Identification of recombinant plasmids containing either Rila or Ald inserts

Recombinants containing either the Rila or the Ald DNA fragments were identified by BamHI and KpnI digests as described in section 2.2.4. The BamHI and KpnI digestions (Fig. 2.4.b) of the successful recombinants produced linearised plasmid DNA and a 1.252 kbp fragment for pFb7mu200 containing the Rila fragment (lane 3) and a 1.243 kbp fragment for pFb7mu200 containing the Ald fragment (lane 4). A 1.093 kbp fragment was produced by BamHI and KpnI digestion of pFb7mu200 (lane 2) with no insert (Fig. 2.4.a and b). The recombinant containing the Rila DNA fragment was designated pFb7mu200-R and the recombinant containing the Ald DNA fragment was designated pFb7mu200-A. It was now possible to construct the dual recombinant after the pFb7mu200-R construct was identified as pFb7mu200-R would now serve as the vector in the strategy used to construct the dual recombinant.
AHSV VP7 AHSV VP7
pFastbac backbone

XbaI

Rila PCR product
33 bp core epitope sequence

AHSV VP7

↓

SaII

gp41 flanking regions

gp41 flanking regions

↓

XbaI

AHSV VP7

↓

SaII

Ald PCR product
18 bp core epitope sequence

↓

XbaI

gp41 flanking regions

gp41 flanking regions

↓

SaII

69 bp

69 bp

66 bp

78 bp

171 bp

162 bp

44
Ligate XbaI and SalI digested Rila and Ald PCR products to pFb7mu200 XbaI and SalI digested vector.

Figure 2.3.: Construction of pFb7mu200-R and pFb7mu200-A. The Rila and Ald DNA fragments were digested with XbaI and SalI and ligated to XbaI and SalI digested pFb7mu200. The plasmid pFb7mu200-R contains the DNA fragment that encodes the RVLAIERYLKD epitope and its 138 bp flanking regions. The plasmid pFb7mu200-A contains the DNA fragment that encodes the ALDSWK epitope and its 144 bp flanking regions.
Figure 2.4: (a) Illustration of BamHI and KpnI restriction enzyme digestion of pFb7mu200. (b) A 2% agarose gel electrophoresis of BamHI and KpnI restriction enzyme digestions of pFb7mu200-R containing the Rila DNA fragment and pFb7mu200-A containing the Ald DNA fragment.

Lane 1: DNA molecular weight marker ФX 174.
Lane 2: Digested pFb7mu200 vector.
Lane 3: Digested pFb7mu200-R.
Lane 4: Digested pFb7mu200-A.
2.3.3 Construction and identification of a dual recombinant pFb7mu200 plasmid containing both Rila and Ald DNA fragments

The dual recombinant containing an insert that encodes a RVLAIERYLKD and ALDSWK fusion protein was constructed as described schematically in Fig. 2.5. In this case the construct that contained the Rila DNA fragment (pFb7mu200-R) was used as the vector and the Ald PCR product encoding ALDSWK and its flanking regions as the insert. Directional cloning was achieved using EcoRI and SalI restriction enzyme digests. The Ald DNA fragment is inserted via the EcoRI and SalI restriction enzyme sites between the 3’ end of the Rila fragment (of pFb7mu200-R) and amino acid 201 of VP7. The dual insert will increase the size of the VP7 protein with 101 amino acids. Both insert and vector were digested with EcoRI and SalI as described in section 2.2.4. The vector was dephosphorylated before the ligation reaction was performed in a 1 : 7 vector to insert ratio as described in section 2.2.6 and 2.2.7. XL1-Blue cells were transformed and plated out onto Tet + Amp + agar plates and incubated overnight at 37°C. Plasmid DNA was isolated from selected colonies using the protocol described in section 2.2.2.

The dual recombinant was identified by BamHI and KpnI digestion (Fig. 2.6) which yielded linearised plasmid DNA and was expected to release a fragment 1.393 kbp in size (lane 6) compared to the fragment size released by the digested pFb7mu200 control of 1.093 kbp and the pFb7mu200-R control of 1.252 kbp. The dual recombinant was digested using a lesser DNA concentration than the controls which could lead to the feint 1.393 kbp fragment observed. The vector used in the construction of the dual recombinant, pFb7mu200-R was used as a control as well as the unmodified pFb7mu200. The digestions of both controls serve to convey the size increase of the dual recombinant as well as to confirm the presence of both the Rila and Ald insert. The dual recombinant containing the nucleotide sequences coding for both RVLAIERYLKD and ALDSWK was designated pFb7mu200-RA.

The presence of the Rila and Ald DNA fragments in all three constructs namely pFb7mu200-R, pFb7mu200-A and pFb7mu200-RA were also confirmed with PCR using primers P1 and P2 (refer to Tables 1 and 2). The amplification products of the recombinants were compared to the control, pFb7mu200 as shown in Fig. 2.7. This figure shows the expected size increases from the control pFb7mu200 (1.344 kbp, lane 5) to the single recombinants pFb7mu200-R (1.503 kbp, lane 2), pFb7mu200-A (1.494 kbp, lane 3) to the dual recombinant pFb7mu200-RA (1.653 kbp, lane 4).
HIV-1 subtype C gp160 sequence, DU 151 (2538bp)

**gp41**

**gp120**

epitopes

Enlarged

RVLAIERYLKD ALDSWK epitope nucleotide sequence

**gp41**

A1 + A2 primers

**gp41**

Ald PCR product

AHSV VP7 pFastbac backbone

Rila

**EcoRI**

**SalI**

162 bp

**EcoRI**

**SalI**

pFb7mu200-R vector

6047 bp

Ligate EcoRI and SalI digested vector and insert

Figure 2.5: A flowdiagram of the construction of the dual recombinant, pFb7mu200-RA, containing both RVLAIERYLKD and ALDSWK epitopes and flanking regions. The pFb7mu200-R and the Ald DNA fragment were digested with EcoRI, SalI and ligated.
Figure 2.6: 2% Agarose gel electrophoresis of the restriction enzyme digestions of the pFb7mu200-RA construct using BamHI and KpnI. The pFb7mu200-RA digestions were compared to the digestions of the controls pFb7mu200 and pFb7mu200-R.

Lane 1: 100 bp ladder DNA Molecular weight marker (Promega).
Lane 2: Uncut pFb7mu200.
Lane 3: Digested pFb7mu200.
Lane 4: Uncut pFb7mu200-R.
Lane 5: Digested pFb7mu200-R.
Lane 6: Digested pFb7mu200-RA.
Figure 2.7: 2% Agarose gel electrophoresis of the PCR products obtained of the different constructs in lanes 2-5 amplified with the P1 and P2 primers (Table1) that flank the insertion site created between amino acid 200 and 201 in VP7.
Lane 1: DNA molecular weight marker MWII and ФX 174.
Lane 2: PCR product after amplification of pFb7mu200-R.
Lane 3: PCR product after amplification of pFb7mu200-A.
Lane 4: PCR product after amplification of pFb7mu200-RA.
Lane 5: PCR product after amplification of pFb7mu200.
2.3.4 Sequence analysis

The constructs were sequenced to confirm the correct assembly of the recombinant constructs. The primers used in the sequencing strategy are outlined in Fig. 2.8. The P1 and P2 primers flank the complete VP7 sequence and target the pFastbac1 sequences (Table 1). The internal D1 and H2 primers flank the MCS created within VP7. The combination of the P1, P2, D1 and H2 primers yielded overlapping sequences which were used to generate a complete sequence of the modified VP7 and its insert (Fig. 2.8). Sequences were generated using the primers in sequencing reactions in automated DNA sequencing described in section 2.2.9. In order to detect any mistakes due to mis-incorporation by Taq polymerase or frame shift mutations, sequencing results were compared to the initial sequence information received from the NICD and available on the NCBI database. Appendix A contains Clustal X sequence alignments of the various constructed recombinants compared to the control pFb7mu200. The sequences confirmed the insertions of the amplified HIV epitopes and their flanking regions as well as the unique EcoRI restriction enzyme site (coding for aa EF). No frame shifts or mutations occurred during the construction of the various recombinant plasmids. The sequence information can also yield information on the predicted hydrophilicity using an appropriate algorithm.

![Sequence Analysis Diagram](image)

Figure 2.8: Schematic representation of the target sequences of the P1 and P2 pFastbac1 specific primers and the D1 and H2 VP7 specific primers on the control pFb7mu200.
2.3.5 Hydrophilicity

Two characteristics of the inserted peptide can potentially influence particle structure. The first of these being the size of the insert and the second the hydrophilic character. The hydrophilic character of the different epitopes were investigated using the algorithm available in Antheprot (Geourjon et al., 1991; Geourjon and Deleage, 1995) to generate hydrophilic profiles of the different proteins based on their amino acid sequences.

The hydrophilic character of each insert is described in Table 4. The hydrophilic profiles of the constructs are displayed in Fig. 2.9 b-d with the inserts highlighted. All the epitopes have a nett hydrophobic character as shown in Table 4. However, there are differences in the level of hydrophobicity displayed by each epitope. As listed in Table 4 the shorter Ald insert has the lowest hydrophobic amino acid character (-14.9) followed by the Rila insert (-20.2). The Rila and Ald dual insert has the highest hydrophobic amino acid character (-35.1). It is unknown what effect the different hydrophobic characters of the inserts will have on the VP7mu200 or VP7mu177 particle, the investigation of which will require sedimentation analysis and Scanning Electron Microscopy of the expressed fusion proteins.

Table 4: Hydrophilic amino acid values of the HIV epitopes according to the parameters proposed by Hopp and Woods (1981).

<table>
<thead>
<tr>
<th>Insert</th>
<th>Rila</th>
<th>Ald</th>
<th>Rila and Ald</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epitope and flanking regions thereof</td>
<td>RVLAIERYLKD 53 aa</td>
<td>ALDSWK 50 aa</td>
<td>RVLAIERYLKD + ALDSWK 101 aa</td>
</tr>
<tr>
<td>Chimeric protein</td>
<td>VP7mu200-R</td>
<td>VP7mu200-A</td>
<td>VP7mu200-RA</td>
</tr>
<tr>
<td>Hydrophilic values</td>
<td>+26.7</td>
<td>+37.2</td>
<td>+61.4</td>
</tr>
<tr>
<td>Hydrophobic values</td>
<td>-46.9</td>
<td>-52.1</td>
<td>-96.5</td>
</tr>
<tr>
<td>Total</td>
<td>-20.2</td>
<td>-14.9</td>
<td>-35.1</td>
</tr>
</tbody>
</table>
Figure 2.9: Hydrophilicity profiles generated by Antheprot (Geourjon et al., 1991; Geourjon and Deleage, 1995).

(a) VP7mu200 - Site 200 is highlighted.
(b) VP7mu200-R – Site 200 and insert highlighted.
(c) VP7mu200-A – Site 200 and insert highlighted.
(d) VP7mu200-RA – Site 200 and insert highlighted.
2.3.6 Baculovirus expression of chimeric VP7-HIV proteins

The baculovirus expression system was used to express the fusion proteins which will be analysed to determine the effect of the different inserts on the VP7 particle. The pFastbac1 transfer vector containing the recombinant VP7 genes were used to generate recombinant bacmid DNA by transforming DH10Bac cells, as described in section 2.2.11. Transposition of the recombinant VP7 gene occurs from the transfer vector into the baculovirus genome (present in the DH10Bac cells). The cells were plated out onto Kan⁺ Gen⁺ Tet⁺ agar plates containing X-gal and IPTG and incubated for 48 hours at 37°C. Blue-white selection was used to differentiate between non-recombinant and recombinant bacmid containing colonies. Recombinant candidate bacmid DNA was isolated using the method described in section 2.2.12. Special precautions were taken to ensure the integrity of the high molecular weight bacmid DNA. Samples were not mechanically stressed by vortex or excessive pipetting. Only fresh samples were prepared for use in a laminar flow hood to prevent contamination of DNA used for transfection.

Recombinant bacmids were identified via PCR using primers P1 and P2 (Table 1) as described in section 2.2.5 Table 2. PCR products (Fig. 2.10) in the size regions corresponding to the expected amplified products of 1503 bp (for pFb7mu200-R in lane 2), 1494 bp (for pFb7mu200-A in lane 3) and 1653 bp (for pFb7mu200-RA in lane 4) were obtained which confirmed the presence of recombinant bacmids. The PCR product in Fig. 2.10 lane 5 was amplified from the vector pFb7mu200 that contained no insert, showed a band which corresponded to the expected size of 1344 bp. The size difference between the vector control and recombinant bacmids was clearly visible on the gel.

Recombinant bacmid DNA identified by PCR were used for transfection to produce recombinant baculoviruses.

Recombinant bacmid DNA containing the transposed pFb7mu200-R, pFb7mu200-A and pFb7mu200-RA sequences were used to transfect SF9 insect cells using Cellfectin™ as described in section 2.2.14. The supernatant containing recombinant baculovirus was harvested and used to prepare a viral stock. VP7mu200 refers to the protein expressed by the recombinant baculovirus after transfection with bacmid containing the pFb7mu200 sequence (Table 3). VP7mu200-R refers to the protein expressed by the recombinant baculovirus after transfection with bacmid containing the pFb7mu200-R sequence. VP7mu200-A refers to the protein expressed by the recombinant baculovirus after transfection with bacmid containing the pFb7mu200-A sequence.
Figure: 2.10: 1% Agarose gel electrophoresis of PCR products of recombinant Bacmid isolates using primers P1 and P2 (see Table 1).
Lane 1: Molecular size markers MWII and Φx 174.
Lane 2: PCR product of bacmid pFb7mu200-R.
Lane 3: PCR product of bacmid pFb7mu200-A.
Lane 4: PCR product of bacmid pFb7mu200-RA.
Lane 5: PCR product of bacmid pFb7mu200.
VP7mu200-RA refers to the protein expressed by the recombinant baculovirus after transfection with bacmid containing the pFb7mu200-RA sequence.

To confirm that the recombinant baculoviruses did express the proteins of interest, SF9 cell monolayers were infected with the VP7mu200, VP7mu200-R, VP7mu200-A and VP7mu200-RA recombinant baculovirus stocks respectively (section 2.2.15) and analysed by SDS-PAGE protein electrophoresis (Fig. 2.11). The proteins VP7mu200-R and VP7mu200-A electrophorese at a position corresponding to ± 42 kDa. The VP7mu200-RA protein electrophoreses at a position corresponding to ± 45 kDa. The VP7mu200-R, -A and –RA proteins show the expected size increase compared to the unmodified protein, VP7mu200 (39 kDa). Expression levels of VP7mu200-R, VP7mu200-A and VP7mu200-RA are slightly less compared to VP7mu200; however expression does not appear to be significantly hampered by the insertion of 50, 53 or 101 amino acids. Sedimentation analysis and Scanning Electron Microscopy was used to further study the chimeric VP7 particle.
Figure 2.11: 12% SDS-PAGE protein electrophoresis of protein expression in SF9 cells using a baculovirus expression system. Insect cells were either uninfected or infected with recombinant or wt baculoviruses.
Lane 1: Rainbow protein size marker.
Lane 2: Mock infection.
Lane 3: Wt baculovirus infection.
Lane 4: Recombinant baculovirus expressing VP7mu200.
Lane 5: Recombinant baculovirus expressing VP7mu200-R.
Lane 6: Recombinant baculovirus expressing VP7mu200-A.
Lane 7: Recombinant baculovirus expressing VP7mu200-RA.
2.3.7 The analysis of VP7 chimeric particles by means of a sucrose density gradient

Sucrose density centrifugation was used to compare the effects of the inserts on the VP7 structure and particle assembly between the VP7mu177 and VP7mu200 chimeras. A schematic representation of the mutant 177 site located in the AHSV VP7 top domain is shown in Fig. 2.12. The VP7mu177 chimeras were constructed with the identical HIV epitopes inserted between the Hind III and Sal I sites using the same cloning strategy (Fig. 2.3 and 2.5) by Rutkowska (2002). The VP7mu177 HIV epitope inserts are identical in size and sequence to the epitopes used in VP7mu200 and were successfully cloned and expressed (Rutkowska, personal communication). A SDS-PAGE electrophoresis comparison in Fig. 2.13 shows the VP7-HIV mu177 and mu200 chimeric proteins to be of the same size as expected. The samples were obtained from pellets after sucrose density centrifugation which also showed the difference in aggregated pelleted particles of the chimeras. VP7mu200 had the most aggregated particles compared to VP7mu177 and the chimeras as it’s the most hydrophobic and similar to unmodified VP7 in its sedimentation profile (Maree, 2000).

Identical sucrose gradients were performed on the VP7mu200 and VP7mu177 chimeras. SF9 cell monolayers were infected with recombinant baculoviruses and harvested 96 hpi to obtain the chimeric proteins. The cell lysates were subjected to centrifugation on a 50 – 70 % discontinuous sucrose gradient as described in section 2.2.20 and fractionated. Each fraction was diluted with 1 x PBS and subjected to centrifugation to separate the hydrophobic particles from the sucrose. The supernatant was discarded and the pellet resuspended in 1 x PBS. The proteins in each fraction was analysed on SDS-PAGE gels (Fig. 2.14 - 2.17). The SDS-PAGE gels were analysed using Quantity One® 1-D analysis software supplied with the Versadoc system (Biorad). The protein content is indicated on the graphs as the % adjusted volume.

VP7mu200 and VP7mu177 particles showed a similar profile as the bottom fractions 1-3 and the top fractions 10-12 contained the most particles (Fig. 2.14 a and b). Both have the majority of particles in fractions 1-4 present as large particulate structures in the gradient. Both showed a decrease in particle size and density in fractions 5-8. Fractions 9-12 showed an increase in particles for both VP7mu177 and VP7mu200 which represent the small particles present in the gradient with decreasing density. However, there are differences in the amount of particles present in the fractions between VP7mu200 and VP7mu177. VP7mu200 had 40 % of its particles present in fractions 1-3 and 43 % in fractions 9-10. VP7mu177 contained approximately 1% of its particles in fraction 1 and 60 % in fractions 2-3 and 25 % in fractions 9-10 with the majority being in fraction 10. The lack of particles in
fractions 9, 11 and 12 could possibly be due to insufficient centrifugation to pellet the smaller particles however smaller VP7mu200 particles did pellet during every repeat experiment performed which casts doubt on this theory but TCA precipitation of the proteins would have been a wiser choice. VP7mu177 tend to be present as large particles rather than smaller particles while VP7mu200 has larger particles. VP7mu200 displayed more smaller particles than VP7mu177. This is possibly a reflection of the difference in sedimentation profile between the different insertion sites created within VP7 where VP7mu177 had its hydrophilic RGD loop extended versus VP7mu200 whose sedimentation profile resembles unmodified VP7 (Maree, 2000). Both VP7mu200 and VP7mu177 (not shown) showed other different sized protein bands which could possibly be cellular debris.

VP7mu177-A had the majority of particles (Fig. 2.15.b) in fractions 1-4 as large particulate structures in the gradient. Less particles were present in fractions 9-12 which contained small particles) compared to VP7mu177. VP7mu177-A showed more particles present in fractions 1,4-9,11 and 12 compared to VP7mu177. An increase in the presence of larger VP7mu177 particles was seen due to the hydrophobic nature of the foreign peptide used.

VP7mu200-A particles were present as different sized particles (Fig. 2.15.a). A decrease in large particles was observed. The majority of VP7mu200-A particles were present as smaller particles varying in size and density (fractions 5-8) with a decrease in small particles (fractions 9-12). The VP7mu200-R (Fig. 2.16.a) distribution profile differs from VP7mu177-R (Fig. 2.16.b) and VP7mu200 but is similar to VP7mu200-A with the majority of particles varying in size across the density gradient. VP7mu177-R displayed a tendency to form large particles with a decrease in smaller particles compared to VP7mu177-A and VP7mu177. The foreign peptide in VP7mu177-R is more hydrophobic than the foreign peptide in VP7mu177-A. The same is true for the peptides inserted in VP7mu200-R and VP7mu200-A respectively. This may influence their ability to form large particles.
Figure 2.12: Schematic representation of the AHSV VP7mu177 vector.

Figure 2.13: 12 % SDS-PAGE electrophoresis comparison of chimeric VP7mu200 and VP7mu177 protein sizes obtained from pellets after sucrose density gradient centrifugation.
Lane 1: Mock infection.
Lane 2: Wild type Baculovirus infection.
Lane 3: Rainbow protein size marker.
Lane 4: VP7mu177.
Lane 5: VP7mu200.
Lane 6: VP7mu177-R.
Lane 7: VP7mu200-R.
Lane 8: VP7mu177-A.
Lane 9: VP7mu200-A.
Lane 10: VP7mu177-RA.
Lane 11: VP7mu200-RA.
The majority of VP7mu200-RA (Fig. 2.17.a) and VP7mu177-RA (Fig. 2.17.b) particles are present in fractions 2-4 and 2-3 respectively. The profile of VP7mu200-RA and VP7mu177-RA in a sucrose density gradient is similar to each other and the VP7mu200 and VP7mu177 particles as most were present as large or smaller particles. VP7mu177-RA and VP7mu200-RA were capable of assembling into large particles however both do not tend to have particles in fraction 1. The VP7mu177-R and –A particles were also capable of assembling into large particles. The capability of VP7mu200-R and –A to assemble into large particles seem to be affected. It is possible that the single inserts which are less hydrophobic than the dual insert may affect particle assembly as represented by the altered distribution in a sucrose density gradient by VP7mu177-A and VP7mu177-R particles (with increased particles in fractions 5-8) or more severely as in VP7mu200-A and VP7mu200-R. It is also possible that the particles of interest associated with lipid cell material which could reduce density which would account for the different sized particles spread across the density gradient.

Lipid and chimeric particle association was investigated by comparing detergent treated samples to untreated samples. Detergent treated samples were prepared as described in section 2.2.20. Untreated samples were prepared as described in section 2.2.21. Briefly the same methods were followed for harvesting and centrifugation. Cell lyses was achieved with detergent in one group and cell lysis was achieved by dounce homogenization in another group. The detergent treated samples are represented by Fig. 2.14.a – 2.17.a. The particle size distribution in a sucrose density gradient has been discussed in the above paragraphs. Dounce homogenization of samples showed less particles in fractions 9-12 of VP7mu200, -A and –RA (Fig. 2.14.c – 2.17.c) compared to detergent treated protein (Fig. 2.14.a – 2.17.a). The majority of particles (76 %) were present between fractions 1-4 for untreated VP7mu200 samples and 46 % for detergent treated VP7mu200 (Fig. 2.14.c) with 30 % of particles in fractions 9-12 for detergent treated and 2 % for untreated VP7mu200. Untreated VP7mu200 particles concentrated in fraction 4 as these seem to be the predominant density and size of these particles. It could be possible that VP7mu200 particles are inclusion bodies and that with mechanical homogenization this association whether it be lipid or protein based was disrupted thereby altering the density of the particle.

The VP7mu200-A sample obtained from mechanical lysis by dounce homogenization (Fig. 2.15.c) had a dominant particle size group of fractions 5 and 7 whereas detergent treated VP7mu200-A showed heterogeneous particle sizes however, of these 50 % of the particles were present as large particles. Detergent treated VP7mu200-A showed 10 % of particles in fractions 9-12 while untreated VP7mu200-A showed 5 % of particles present in these fractions. As both The untreated VP7mu200-R sample (Fig. 2.16.c) showed a dominant
particle grouping of fractions 5-10. Approximately 72% of untreated VP7mu200-R particles were present in fractions 5-7. compared to 51% of particles in the same fractions for detergent treated VP7mu200-R. Detergent treated VP7mu200-R contained 22% of its particles in fractions 9-12 while untreated VP7mu200-R showed 20%. Mechanically lysed VP7mu200-RA (Fig. 2.17.c) showed most particles to be present in fractions 4-7 (90%) compared to detergent treated VP7mu200-RA with 45% of particles present in fractions 1-4 and 37% present in fractions 9-12. In all the untreated chimeras and VP7mu200, less particles were present in fractions 9-12 compared to detergent treated chimeras. However, the untreated chimeras showed a decrease in particles in fractions 1-4 compared to the detergent treated chimeras while untreated VP7mu200 showed an increase to 76% of particles present in fractions 1-4 compared to 46% for detergent treated VP7mu200. Mechanical lysis via homogenization may disrupt VP7 and lipid association with cell debris into smaller sized, less aggregated structures resulting in no observable particles in the pellet compared to detergent treated particles (Fig. 2.18. a.i-ii).

Overall, detergent treated particles showed more size differences on the graphs than in the absence of detergent with mechanical lysis. This seems to suggest that the different sized particles observed with the use of detergent were subjected to insufficient lysis or removal of cellular debris. These large associations with VP7 particles were unstable as they were easily disrupted mechanically which showed VP7 particles to lie further away from the bottom of the gradient than detergent treated samples showed. The detergent treated particles may have had associated lipid material thereby decreasing their density leading to the differently sized particles spread across the density gradient.

Fig. 2.18.b showed proteins from a wt baculovirus infection of SF9 cells obtained after mechanical lysis. The majority of proteins are present in fractions 5-12. Comparison of Fig. 2.18.b to the mechanically lysed chimeric proteins show the contaminating protein bands on these protein electrophoresis gels to be baculovirus infected cell debris. Incomplete cell lysis occurred due to the lack of lysis buffer in the homogenization step which resulted in large fragments of cellular material. This can be seen when comparing the detergent treated samples of the proteins to the untreated samples. The cell debris was not separated by low speed centrifugation after homogenization resulting in cytoplasmic and nuclear cytoskeletal fractions being separated via differences in size and density with the proteins of interest.

It is possible that proteases and a poorer protein expression of VP7mu200-R may be responsible for the difference in concentration of foreign protein bands seen in untreated VP7mu200-R compared to the other untreated chimeras. Protease inhibitors should also be included in the protocol. In conclusion detergent treatment and dounce homogenization
coupled with the separation of cytoplasmic and nuclear cytoskeletal fractions will yield a protein sample with less cellular debris. Only the insoluble particles were analysed due to the removal method employed to separate the sample from the sucrose. We do not know how much of the chimera is soluble and insoluble and how this would differ between the different chimeras.
Figure 2.14: Particle distribution of VP7mu200 and VP7mu177 in a 50 – 70 % discontinuous sucrose gradient. Lane 1-12 contains sucrose gradient fractions 1 to 12. (a)i) 12 % SDS-PAGE electrophoresis of detergent treated VP7mu200. (a)ii) VP7mu200 particle distribution graph. (b)i) 12 % SDS-PAGE electrophoresis of VP7mu177. (b)ii) VP7mu177 particle distribution graph (Courtesy of D. Rutkowska). (c)i) 12 % SDS-PAGE electrophoresis of untreated VP7mu200. (c)ii) VP7mu200 particle distribution.
Figure 2.15: Particle distribution of VP7mu200-A and VP7mu177-A in a 50 – 70 % discontinuous sucrose gradient. Lane 1-12 contains sucrose gradient fractions 1 to 12.

(a)i) 12 % SDS-PAGE electrophoresis of detergent treated VP7mu200-A. (a)ii) VP7mu200-A particle distribution graph. (b)i) 12 % SDS-PAGE electrophoresis of VP7mu177-A. (b)ii) VP7mu177-A particle distribution graph (Courtesy of D. Rutkowska). (c)i) 12 % SDS-PAGE electrophoresis of untreated VP7mu200-A. (c)ii) VP7mu200-A particle distribution graph.
(a) Detergent treated

(a)ii) VP7mu200-R particle distribution

(b)ii) VP7mu177-R particle distribution

(c) Untreated

(c)ii) VP7mu200-R particle distribution

Figure 2.16: Particle distribution of VP7mu200-R and VP7mu177-R in a 50 – 70 % discontinuous sucrose gradient. Lane 1-12 contains sucrose gradient fractions 1 to 12. (a)ii) 12 % SDS-PAGE electrophoresis of detergent treated VP7mu200-R. (a)ii) VP7mu200-R particle distribution graph. (b)ii) 12 % SDS-PAGE electrophoresis of VP7mu177-R. (b)ii) VP7mu177-R particle distribution graph (Courtesy of D. Rutkowska). (c)ii) 12 % SDS-PAGE electrophoresis of untreated VP7mu200-R. (c)ii) VP7mu200-R particle distribution graph.
Figure 2.17: Particle distribution of VP7mu200-RA and VP7mu177-RA in a 50 – 70 % discontinuous sucrose gradient. Lane 1-12 contains sucrose gradient fractions 1 to 12.

(a)i) 12 % SDS-PAGE electrophoresis of detergent treated VP7mu200-RA. (a)ii) VP7mu200-RA particle distribution graph. (b)i) 12 % SDS-PAGE electrophoresis of VP7mu177-RA. (b)ii) VP7mu177-RA particle distribution graph (Courtesy of D. Rutkowska). (c)i) 12 % SDS-PAGE electrophoresis of untreated VP7mu200-RA. (c)ii) VP7mu200-RA particle distribution graph.
Figure 2.18.a: Particle distribution of detergent treated VP7 chimera infected samples and untreated VP7 chimera infected samples subjected to centrifugation on a 50 – 70 % discontinuous sucrose density gradient. (a)i) Chimeric protein pellets obtained after 50 – 70 % discontinuous sucrose gradient density centrifugation of chemically lysed samples. Mechanically lysed particles pellets are not shown as bands were not visible with Coomassie staining and subsequent Quantity One® 1-D analysis.

(a)ii Comparison of detergent treated vs untreated cells (in which cell lysis achieved with dounce homogenization). Each lane contains two colour coded bars which are described in the legend.

Lane 1- Detergent treated VP7mu200 infected cells.
Lane 2- Untreated VP7mu200 infected cells.
Lane 3- Detergent treated VP7mu200-A infected cells.
Lane 4- Untreated VP7mu200-A infected cells.
Lane 5- Detergent treated VP7mu200-R infected cells.
Lane 6- Untreated VP7mu200-R infected cells.
Lane 7- Detergent treated VP7mu200-RA infected cells.
Lane 8- Untreated VP7mu200-RA infected cells.
Figure 2.18: (b) 12 % SDS-PAGE protein electrophoresis of wild type Baculovirus infection of SF9 cells. Dounce homogenized cells were subjected to centrifugation on a 50 – 70 % discontinuous sucrose gradient. Lane 2-13 contains sucrose gradient fractions 12 to 1.
2.3.8 Scanning Electron Microscopy

The effect of inserting 50, 53 or 101 amino acids in VP7 on the crystalline structure of VP7mu200 was investigated by Scanning Electron Microscopy and compared to the chimeric VP7mu177 proteins.

Recombinant proteins were harvested from recombinant baculovirus infected cells and treated as described in sections 2.2.19, 2.2.20 and 2.2.23. Briefly, cells were harvested 96 hpi, lysed and purified on a 50 - 70 % discontinuous sucrose gradient. The fractions containing the particles of interest (fractions 1-4) were collected by centrifugation and prepared for Electron Microscopy.

The unmodified VP7mu200 vector (Fig. 2.19.c) typically yielded hexagonal crystalline structures observed previously with unmodified AHSV VP7 (Maree, 2000). Crystalline structures were observed in recombinant baculovirus infected insect cells typically reported for unmodified AHSV VP7 under the light microscope (result not shown). As can be seen in Fig. 2.19.a, a smooth hexagonal crystal was present in the lower left hand corner with a rosette structure, also of VP7 origin, in the centre. The rosette showed the layers of the crystal exposed to the environment. This rosette structure may be representative of the process of formation of the intact, smooth surfaced crystal (Fig. 2.19.a and b). This rosette could also represent several VP7 crystals layered on top of each other forming an aggregate.

The VP7mu200-R recombinant Fig. 2.19.d-f yielded typically flat protein structures ±10 µm in diameter that corresponds to the size range of approximately 8-10 µm previously observed by colleagues (Maree, 2000; Van Rensburg, 2004). The flat structures were slightly rounded with a rough to layered surface. The characteristic hexagonal shape as well as crystal formation (visible under light microscopy) was abolished. The VP7mu200-A particles (Fig. 2.19.g-i) and the VP7mu200-RA particles (Fig. 2.19.j-l) exhibit the same features as the VP7mu200-R particles. The shape and layering of these recombinant proteins are tough with no definitive shape and doesn’t resemble the rosette structure in Fig. 2.19.a of VP7mu200.
Figure 2.19: Scanning Electron Microscopy photographs of the chimeric protein particles formed by (a-c) VP7mu200 and (d-f) VP7mu200-R (The magnification value is indicated in the heading of each photo).
Figure 2.19: Scanning Electron Microscopy photographs of the chimeric protein particles formed by (g-i) VP7mu200-A and (j-l) VP7mu200-RA (The magnification value is indicated in the heading of each photo).
An initial comparison demonstrated characteristically large crystals produced by VP7 expression in insect cells visible with VP7mu177 and VP7mu200 under the light microscope (results not shown). The chimeric proteins of both VP7mu177-HIV and VP7mu200-HIV did not form these crystalline structures visible under the light microscope (results not shown). The chimeric VP7mu177 proteins were purified as previously described for the chimeric VP7mu200 proteins. In order to compare the different proteins, the same fractions were taken for Scanning Electron Microscopy analysis of the chimeric VP7mu177 proteins as for the chimeric VP7mu200 proteins. The chimeric VP7mu200 particles have been previously discussed and are shown in Fig. 2.19. The chimeric VP7mu177 particles are shown in Fig. 2.20. Both VP7mu200 and VP7mu177 produced flat hexagonal crystals (Fig. 2.19.a and Fig. 2.20.a) as reported for unmodified AHSV VP7 (Maree, 2000). Both the VP7mu200 and VP7mu177 chimeric particles appeared slightly rounded with a rough surface. In all cases the structures were disrupted from the initial particles formed by the VP7mu200 and VP7mu177 proteins. The initial structural disruption appears to be stable in VP7mu200-HIV and VP7mu177-HIV from inserts of 50 to 101 aa. The effect of the structural disruption on the recognition of the inserts by an HIV serum is unknown.
VP7mu177 based particles

(a) VP7mu177

(b) VP7mu177-A

(c) VP7mu177-R

(d) VP7mu177-RA

Figure 2.20: Scanning Electron Microscopy photographs of the VP7mu177 chimeric particles prepared by D. Rutkowska and provided for comparison between VP7mu200 chimeric particles.

(a) VP7mu177 (X 8000 magnification).
(b) VP7mu177-A (X 3300 magnification).
(c) VP7mu177-R (X 6000 magnification).
(d) VP7mu177-RA (X 5000 magnification).
2.3.9 Western blot analysis

Previous research by colleagues, discussed in the literature review and chapter introduction, has shown that short epitopes lacking flanking regions do not elicit a detectable epitope specific immune response. Flanking regions were incorporated in our strategy to express a fusion protein with a 3D structure capable of eliciting an epitope specific immune response. The fusion proteins were successfully expressed however the particles formed by these chimeras were distorted. We did not know whether this would influence the epitope presentation. A Western blot was performed to investigate the antigenicity of these particles.

Samples of wt baculovirus, mock infections, VP7mu200, VP7mu200-R, VP7mu200-A, VP7mu200-RA, VP7mu177, VP7mu177-R, VP7mu177-A and VP7m177-RA proteins were separated via 12 % SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane as described in section 2.2.24. The wt baculovirus, mock infection and VP7mu200 or VP7mu177 were used as negative controls. The results are presented in Fig. 2.21.a and b. All the VP7mu200 and VP7mu177 chimeric proteins were detected by the primary antibody, HIV-1 subtype C antiserum from infected individuals obtained from Dr. M. Papaphanasopolous (NICD). Non-specific binding occurred on both the VP7mu177 and VP7mu200 Western blots due to insufficient blocking of irrelevant protein binding sites. The polyclonal antiserum used could have been diluted more to reduce non-specific binding and more vigorous washing could also have been included. The RVLAIERYLKD and ALDSWK epitopes are antigenic as the epitopes were recognized by the HIV-1 subtype C serum. Furthermore the epitopes are antigenic in both site 177 and 200.

The level of the HIV specific immune response was not quantified between the different VP7mu200 and VP7mu177 chimeras as immunization experiments and ELISA tests will need to be done. That will indicate which VP7 mutant site is most effective at presenting foreign epitopes to the immune system. We included a negative control in the analysis however the pFastbac gp160 clone obtained from NICD will be useful as a positive control once transposition and transfection have taken place. During this study we continually selected the insoluble material based on the method used to separate the sucrose from the material by dilution and low speed centrifugation. We therefore do not know if the soluble fractions contain antigenic chimeric material.
Figure 2.21: Western blot analysis of chimeric VP7mu200 and chimeric VP7mu177 proteins. The epitopes RVLAIERYLKD and ALDSWK were detected by a HIV-1 subtype C antiserum from infected individuals obtained from Dr M. Papaphanasopolous (NICD).

(a) VP7mu200 Western blot.
Lane 1: Rainbow protein size marker.
Lane 2: VP7mu200-RA.
Lane 3: VP7mu200-A.
Lane 4: VP7mu200-R.
Lane 5: Wt baculovirus infection.
Lane 6: Mock infection.

(b) VP7mu177 Western blot (courtesy of D. Rutkowska).
Lane 1: Rainbow protein size marker.
Lane 2: VP7mu177-A.
Lane 3: VP7mu177-R.
Lane 4: VP7mu177-RA.
Lane 5: VP7mu177.
2.4 Discussion and concluding remarks

Several AHSV-9 VP7 vectors with different inserts were made as previously discussed in the Literature review. Up to the time of this investigation no comparison had been made between inserting foreign epitopes in the different cloning sites engineered on the VP7 protein. Currently 4 AHSV VP7 vectors are available: VP7mu144, VP7mu177, VP7mu200 and a triple vector VP7 144/177/200 (Maree, 2000; Riley, 2003; Van Rensburg, 2004). In order for a vaccine to be effectively used it needs to be investigated to ascertain the effects of insertions on eg. expression, structure, stability, insert size and number of inserts that can be inserted. The characteristics of each need to be assessed to identify a suitable vaccine delivery system. VP7mu200 and VP7mu177 were chosen for the first of many comparison studies between these vectors.

The aims of this study was to compare the structure, stability, the assembly into VP7 particles of the same insert in two different insertion sites in VP7 and the effect of flanking regions on the antigenicity of the insert.

The sequence chosen for the inserts were Rila which encode the RVLAIERYLKD epitope and Ald which encode the ALDSWK epitope of HIV-1 subtype C. The HIV-VP7mu177 chimeras were produced by Daria Rutkowska. The pFb7mu200 recombinants were constructed successfully with these fragments as demonstrated by the restriction enzyme digests, PCR and sequencing results discussed in section 2.3.1 – 2.3.4. Successful expression was observed in all three chimeras namely VP7mu200-R, VP7mu200-A and VP7mu200-RA. The effect of these various inserts on particle structure and stability was investigated by sedimentation analysis and Scanning Electron Microscopy and compared to the VP7mu177 chimeras.

Molecules which differ in size, shape and density have different sedimentation profiles under non equilibrium centrifugation conditions. Information with regards to the particle assembly process can be obtained as multimeric particles would differ in their sedimentation profiles through various assembly stages. Small particles are slow sedimenting particles and large particles are fast sedimenting particles and would thus move further in the gradient (Cooper, 2000; Yeaman, 2003; Schuck, 2004).

The chimeric proteins were subjected to non equilibrium centrifugation on a 50 – 70 % discontinuous sucrose density gradient. The particle distributions of VP7mu200 and VP7mu177 were similar. The majority of particles were present in fractions 1-4 as large particles and as smaller particles in fractions 9-12. The VP7mu177-A and VP7mu177-R
particle distribution profile was similar to VP7mu177 but with more particles exhibiting size heterogeneity than VP7mu177. The particle distribution of VP7mu200-A is different compared to both VP7mu200 and VP7mu177-A. The particles in various sizes were spread across the gradient in fractions 1-10. The majority of the particles were present in fractions 5-8. Fewer particles assembled into large aggregated structures which dominate the bottom fractions 1-4. The VP7mu200-R particle distribution was similar to VP7mu200-A with fractions 5-8 containing the majority of the particles. VP7mu200-RA and VP7mu177-RA have particle distributions similar to VP7mu200 and VP7mu177.

It was possible that the heterogenous sized particles observed in the gradient fractions could be attributed to cell debris and lipid association. This was investigated by comparing detergent treated VP7mu200 chimera samples with untreated VP7mu200 chimera samples in which cells were lysed by dounce homogenization. The samples were then subjected to sucrose density centrifugation. Untreated VP7mu200 showed small particles in fractions 9-12 to decrease significantly compared to detergent treated VP7mu200. The different peak distributions of detergent treated VP7mu200 vs. untreated VP7mu200 suggest the breakdown of large aggregates (fractions 1-4) to particles which were predominantly present in fraction 4. This suggests that material associated with VP7mu200 was lost with mechanical homogenization which decreased the VP7mu200 particle density. Untreated VP7mu200-A particles on a sucrose gradient showed a bimodal distribution consisting of fraction 5 and fraction 7 respectively. The presence of 2 observational units suggests 2 predominantly sized VP7mu200-A particles. Particles in fraction 5 are denser than particles in fraction 7. The particles in the different fractions may also differ in shape as that can also influence sedimentation profiles (Berg et al., 2002). Further investigation is required to determine if both fractions are from VP7 origin by western blot analysis with AHSV antiserum and a conformation with the HIV antiserum used in this study. The predominant shape of particles in fraction 5 may be compared to fraction 7 (via Scanning Electron Microscopy) to determine if shape may play a role. Untreated VP7mu200-R particles on a sucrose gradient showed particles in various sizes. Untreated VP7mu200-RA particles on a sucrose gradient had a distribution similar to untreated VP7mu200-R and -A.

The 50, 53 and 101 aa inserts in VP7mu200 and VP7mu177 disturbed the structure of VP7. The characteristic hexagonal VP7 crystals were abolished and flat rounded particles with a rough surface were observed using Scanning Electron Microscopy. All the chimeric VP7mu200 and VP7mu177 proteins retained their antigenic conformation regardless of possible particle assembly effects and distortion of the VP7 particle structure as the inserts denatured conformations were recognized by HIV serum in a Western blot.
The results show that the particle distribution differed between VP7mu200-R, VP7mu200-A and VP7mu200-RA. The VP7 crystal is composed of associated trimers. The loose trimers are soluble. The soluble trimers were not included in the experiments due to the centrifugation conditions used in this study. The small particles formed by the association of trimers assemble to form large VP7 particles which are highly hydrophobic and tend to aggregate into large structures as represented by the particles in the bottom fractions of highest density of the gradient. There is a difference in size and the degree of hydrophobicity of the inserts. The VP7mu200-A inserts is less hydrophobic than VP7mu200-R which in turns is less hydrophobic than VP7mu200-RA. The insert used in both VP7mu200-RA and VP7mu177-RA is the most hydrophobic of the inserts used in this study. Particle assembly is not affected by this insert as small particles assemble into large particles present in fractions 1-4. Thus an increase in the hydrophobic character of the Rila insert by adding the Ald insert results in a restoration of the typical assembly process of VP7mu200.

The insertion of 101 aa in both VP7mu200 and VP7mu177 (detergent treated samples) resulted in predominantly large particulate structures. While the inserts of 53 and 50 aa which are less hydrophobic than the dual insert resulted in the increase of smaller particulate structures with VP7mu200. VP7mu177 with the 50 and 53 aa inserts were still capable of forming predominantly large particles. Thus VP7mu177 and 200 particle size distribution on a sucrose gradient differ according to the hydrophilic character of the peptide inserted. Mutant site 200 is displayed in the central area of the trimer (Fig. 1.2). The increased hydrophobicity (VP7mu200-RA) compared to the single chimeras may stabilize the hydrophobic bonds resulting in an increase in assembly into large particulate structures as trimer interactions are non-specific and hydrophobic (Limn et al, 2000). Mutant site 177 is located on the edges of the trimeric molecule (Fig. 1.2) and occurs in a hydrophilic RGD loop (Maree, 2000). The stability of the particles remains as the insertion extends the RGD loop causing less steric hindrance and disruption to hydrophobic bonds.

Small particulate structures also formed with a 25 aa insert of a neutralizing AHSV VP2 epitope in VP7mu144. Riley (2003) proposed that the small particles were due to reduced hydrophobic interactions between trimers which reduced the stability of the large particulate structures (Riley, 2003). The focus has been largely on the large particles at the bottom of the gradient as these have elicited VP7 immune responses (Meiring, 2001; Meyer, 2002). Immunization with small particles (fractions 8-10) by Riley (2003) did not produce a detectable immune response against the epitope. However, large particle aggregates can also be problematic as the epitopes can be shielded from the environment. Thus a difference in the efficiency of antibodies elicited between the large particles formed by VP7mu200-RA
(fractions 1-4) and the smaller particles (fractions 5-8) formed by VP7mu200-R and –A could be possible and should be investigated.

The different peak distributions of detergent treated VP7mu200 vs. untreated VP7mu200 suggest the breakdown of large aggregates (fractions 1-4) to particles predominantly present in fraction 4. This may be due to AHSV VP7’s tendency to aggregate as well as association with lipids or being caught in cellular debris (Basak et al., 1996; Maree, 2000). Unmodified VP7 formed predominantly large particulate structures with highly aggregated particles pelleted during density gradient centrifugation. Sonication of VP7 resulted in distinct size and density structures present in fraction 3 of a 50 - 70 % discontinuous sucrose gradient (Van Rensburg, 2004). Dounce homogenization of VP7mu200 and chimeras presented baculovirus infected cell debris upon SDS-PAGE analysis. Procedures such as homogenization break the plasmamembrane as well as the membrane of the Endoplasmic reticulum into small fragments which can reseal to form small vesicles. Careful homogenization will leave cellular components such as nuclei, lysosomes, peroxisomes and mitochondria largely intact (Cooper, 2000; Alberts et al., 2002). Research has shown that fractionation of mammalian cells on sucrose gradients can separate microsomal (18.3 – 26.3 %), mitochondrial (55.5 – 64.5 %) and nuclear (73.9 – 83.8 %) material (Srinivas et al., 2004). Lysis buffer and separation of cytoplasmic and nuclear cytoskeletal fractions by low speed centrifugation was not included. Thus the cell debris obtained by homogenization was also included in the sedimentation analysis. Cellular debris still present in the untreated samples may still influence results in that optimal homogenization wasn't achieved leaving the original problem of cellular debris aggregating with the particles partially solved and may possibly explain why there is still particles spread across fractions. Western blots on Mini SDS-PAGE gels would aid in proving the contaminating bands to be cellular debris however the large gels has better band separation but is impractical for western blots.

The insertion of the HIV-1 subtype C epitopes may have disrupted the crystalline structure due to weakened hydrophobic interactions as the characteristic hexagonal crystals produced by unmodified VP7 consist of trimers bound by hydrophobic interactions which would result in the rounded, rough structures observed (Basak et al., 1996; Limn et al., 2000). Flat rounded structures were also observed with the VP7 144/177/200 protein but unlike the VP7mu177 and VP7mu200 proteins and their chimeras, chimeric VP7 144/177/200 resembles the VP7 144/177/200 vector particles indicating a more stable vector (Van Rensburg, 2004).
Scanning Electron Microscopy has shown the presence of the characteristic hexagonal crystal as well as the rosettes for unmodified VP7mu200. The rosettes could be representative of the assembly into the crystal or represent the aggregation of several VP7 crystals layered onto one another. Only fractions 1-4 were analysed using Scanning Electron Microscopy as these contained the large particles that would be used in immunization. If we assume that the rosette is an intermediate structure of the assembly process, one could expect this to be the predominant form in fractions 5-12. If the rosette is formed by crystal aggregation it would not be predominantly present in fractions 5-12 as the bottom fractions contain particles with the highest density in the gradient. Analysis of fractions has been limited to the large structures present in the bottom fractions. Analysis of other fractions has been hampered by the presence of contaminating baculovirus protein and as such have been largely neglected.

It is possible that the large particles investigated here may not be effective in eliciting an immune response. HIV infection and spread occurs through mucosal routes. Transportation to the lymph nodes occurs via Dendritic and Langerhans cells (Gray and Puren, 2000; Williamson et al., 2000). If we consider oral vaccine delivery, Peyer’s patches would take up the particles and induce mucosal immune responses. Peyer’s patches have been reported to take up particles of 10 µm and smaller. Larger particles sized between 5-10 µm remained in Peyer’s patches while particles smaller than 5 µm could be systemically transported in the lymph (O’Hagan, 1996; Chew et al., 2003). The hydrophobicity of a particle also affects its uptake as more hydrophobic particles are taken up than hydrophilic particles (O’Hagan, 1996; Maurice et al., 2003). Thus the chimeric VP7mu200 particles has an advantage in its hydrophobicity but may be hampered by its size in eliciting an immune response.

VP7 hexagonal-like crystals were observed in Electron Microscopy studies of VP7mu200 and VP7mu177. Crystals were ± 10 µm in diameter. The insertion of 50, 53 and 101 amino acids did not significantly impair expression of VP7mu200 in insect cells. However no characteristic crystalline structures were observed in the VP7-HIV chimeric baculovirus infected insect cells under light microscopy. The absence of the characteristic hexagonal crystalline structures have also been observed with the analogous VP7mu177 inserts, 25 aa neutralizing AHSV VP2 epitope insert in VP7mu144 and the VP7 144/177/200 vector and inserts (Rutkowska, personal communication; Riley, 2003; Van Rensburg, 2004). The particles observed using Scanning Electron Microscopy show some resemblance to the rosettes. The particle formed may be due to decreased hydrophobic interactions required to assemble into a large crystal thus resulting in incomplete assembly. However, the VP7mu200-RA particle (detergent treated sample) has a similar sedimentation profile than VP7mu200 (detergent treated
sample) but shows the same distorted particle as with the VP7mu200-A and VP7mu200-R particles which suggests that the distortion may also be attributed to steric interference.

It is possible that due to the steric interference and decreased hydrophobic bonds that the particles observed show some dissociation between the sheets that form the flat VP7 crystal that could show as bulges which could be represented by the rough appearance of the particle. In density gradient centrifugation compact particles have a smaller frictional coefficient compared to extended particles with the same mass. The extended particle will sediment slower. The shape of a particle also influences sedimentation as viscous drag is affected (Berg et al., 2002). The hexagonal VP7 shape is affected which could also account for the difference in the sucrose density gradient distribution of the chimeric particles compared to unmodified VP7mu200. However, the VP7mu177 chimeric particles have a slightly similar density gradient distribution to the unmodified VP7mu177 but are present as distorted particles. This contradiction may be partly explained by lipid and cell debris association of the particles which can alter the density of the particles and needs to be investigated. The VP7mu177 chimeric particles should also be subjected to density gradient centrifugation after dounce homogenization of the cells to answer this question.

It is possible that the particles may well be inclusion bodies. The crystal may be an inclusion body with a particular shape. The rosettes could be a distortion of these inclusion bodies. The rosette structure shows several layers to be exposed which could decrease the density of the particle. The structural distortion in the chimeric particles can decrease their density observed on a sucrose density gradient compared to VP7mu200 particles. The density of VP7mu200 and the chimeric particles are decreased by possible lipid or protein association forming an inclusion body resulting in the particle spread across the gradient and is shown by comparing detergent treated and untreated samples subjected to sucrose density centrifugation. Inclusion bodies can be protein aggregates which form due to insufficient folding by the cell’s machinery. This often occurs in recombinant bacteria where plasmid encoded genes are highly expressed (Pearce, 2001). The polyhedrin promoter used to drive foreign gene expression at high levels in the baculovirus expression system can produce more foreign protein than the cell’s folding machinery can cope with. Research into inclusion bodies has shown the in vivo inclusion body production can be caused by a protein aggregation and solubility imbalance. The imbalance lies in the equilibrium between protein precipitation, refolding and solubility. If protein production is halted allowing the cell’s folding machinery to catch up, it can regain the balance between the factors (Pearce, 2001). Inclusion bodies can also occur at sites where assembly of the virion takes place. This can be in the cell nucleus such as with adenoviruses which consists of crystalline virions or viral
particles or cytoplasmic as with the rabies virus which produces intracytoplasmic inclusion bodies that may be accumulated nucleocapsids (Levy et al., 1994).

The purposes of including flanking regions were to investigate the effect of such flanking regions on the immunogenicity of the epitope. This is important as the ALDSWK epitope used in this study is the proposed HIV-1 subtype C equivalent to the ELDKWA epitope in HIV-1 subtype B. Failures in eliciting 2F5 mAbs have been reported by other researchers which used the ELDWA epitope in their vaccine strategies. These failures may be due to a complex epitope structure, discontinuous epitope or continuous epitope features involving longer flanking regions (Zwick et al., 2001; Parker et al., 2001; Menendez et al., 2004). Muster et al. (1995) concluded that the antigenicity of ELDKWA was influenced by C and N terminal amino acids (Muster et al., 1995). Results obtained from affinity selection of a HxBC2 (HIV-1 strain) fragment library with mAb 2F5 suggest that the ELDKWA target recognition involve flanking residues (Zwick et al., 2001). Menendez et al. (2004) also concluded that peptides extending beyond ELDKWA resulted in increased epitope antigenicity. C terminal residues flanking the DKW core epitope (of ELDKWA) were found to be required for high affinity binding. The mAb 2F5 was found to involve 2 epitope regions: the DKW core sequence and the C terminal residues of which binding is multi specific (Menendez et al., 2004).

The peptide sequence LELDKWASL is required for maximum peptide antigenicity with the amino acid sequence NEQELLELDKWASLWN identified by Parker et al. (2001) to be important for recognition by mAb 2F5 (Parker et al., 2001; Menendez et al., 2004). The fact that C and N terminal residues are important for the conformation required for recognition by 2F5 is well documented however the extent of the flanking regions required differs. This is important for future immunogenicity studies with the chimeric proteins produced in this study. Antigenicity problems have already been encountered. Meyer (2002) concluded that the hydrophobic nature and size of the ELDKWA epitope presented by the single VP7mu144 and VP7mu177 and triple VP7mu177 ELDKWA insertions may have been the reason for poor presentation with no ELDKWA specific immune response observed in mice. Larger inserts, such as these used in this study, would guarantee exposure to the immune system. Short inserts with hydrophilic adaptors were also suggested to increase hydrophilicity, exposure and size to ensure display to the immune system (Meyer, 2002).

Another factor influencing immunogenicity is that Wade-Evans et al. (1997) showed the VP7 crystal conformation to be important to elicit protection. Both VP7mu177 and VP7mu200 chimeras should induce an immune response as the epitope was shown to be antigenic by a
Western blot. It is thus possible that the VP7 hexagonal structure is unimportant for foreign epitope display. As long as the foreign peptide does not affect trimer assembly, particle formation occurs, is presented on the VP7 surface and the conformation of the peptide is immunogenic. It is also important to note that in the context of an AHSV vaccine, it is necessary to keep the crystalline structure as it is the conformation shown to be effective in eliciting cellular protection which can be combined with other AHSV epitopes to elicit broad protection against AHSV (Wade-Evans et al., 1997).

In this study we were able to successfully express 50, 53 and 101 aa inserts in the AHSV VP7 vaccine display systems. Site 177 is of particular interest as it is located in the hydrophilic RGD loop (Tan et al., 2001; Maree, 2000). Van Rensburg (2004) was able to express 250 aa in site 177 of the triple VP7 144/177/200 vector without affecting particle formation. Site 177 is able to tolerate large inserts as the inserts extend the loop without affecting the structural integrity of the particle. It is unknown whether site 200 will tolerate a 250 aa insertion as it is less exposed to the surface compared to site 177. The insertion of 250 aa in site 177 compares well with other systems which have been able to express a foreign peptide of 238 aa.

AHSV NS1 which is also being investigated as a vaccine delivery system was able to form tubules with a 150 aa AHSV VP2 insertion. A hydrophilic region used on the C terminal of NS1 is exposed on the surface of the tubule and as such is theoretically capable of incorporating large inserts. The advantage of the NS1 system above VP7 is that the tubules are helical which suggests that NS1 may be capable of incorporating larger inserts than VP7 (de Lange, personal communication; Ghosh et al., 2002a). BTV NS1 has been fused with a maximum of 238 aa expressing GFP as well as a variety of foreign epitopes were successfully expressed such as a 14 aa CD8+ T cell epitope from the Lymphocytic chorio meningitis virus nucleoprotein, the 9 aa Foot and mouth disease virus VP1 and 19 aa of the Influenza a virus HA of which all formed NS1 tubules and were immunogenic (Ghosh et al., 2002a; 2002b). Other vaccine systems have also been able to display large inserts such as those based on the hepatitis B virus nucleocapsid. A number of these will be discussed below.

The hepatitis B virus nucleocapsid (183 aa) consists of a single core protein. The 144 aa N-terminal is the assembly domain while the C terminal is required for nucleic acid binding. Electron Microscopy studies revealed icosahedral particles consisting of 180 and 240 subunits. Insertion into the central c/e1 epitope region located at aa site 80 with the 238 aa GFP protein was made yielding particles which display GFP on their surface. The assembly
domain is predicted to have 4 helices allowing the incorporation of such large inserts. In another experiment the nuclease (17 kDa) of *Staphylococcus* aureus was fused to the C terminal which is located in the interior due to its expected nucleic acid binding function. Particles were formed which were enzymatically active. Beterams et al. (2000) calculated that a maximum of 240 subunits can be packaged inside the particle assuming no packaging problems will occur.

It was calculated as follows: the radius of the inner capsid lumen of the particle is ± 13 nm, for a regular sphere the volume would be 9000 nm$^3$. Native *Staphylococcus* aureus nuclease is ± 4 x 3 x 3 nm with the volume of one subunit as being 36 nm$^3$. Thus 240 *Staphylococcus* aureus nuclease subunits would occupy 8600 nm$^3$ inside the particle (Beterams et al., 2000). Structural flexibility is important for a carrier protein to be able to incorporate large inserts as is its versatility in displaying epitopes.

A prime example of which is hepatitis B based subunit vaccines. The carrier protein HBsAg (25 kDa) was fused to HIV-1 gp120 (95 kDa with a V1V2 deletion) which still formed particles that showed a high affinity for CD4. This carrier protein is highly flexible as it assumes different forms naturally such as dane particles (42 nm), spherical particles (22 nm), rods (22 nm in diameter) as well as hepatitis δ virus virions (32 nm). Another reason why such a large fusion was successful is that the carboxyl end of HBsAg is exposed on the surface of the molecules (Berkower et al., 2004). BTV VP7 crystals grown by Basak et al. (1992) assumed hexagonal rod-shapes, rectangular plate-like and diamond-shaped plates. It is thus possible for the particle shape to be flexible although it is dictated by trimer association. The rounded particles formed by the chimeras may not in itself be an indication of instability however the subunits (trimers) ability to assemble into large particles which can be sonified into several distinctly sized particles may well be.

In conclusion the VP7mu177 and VP7mu200 vectors show promise as vaccine delivery systems with particles formed with a maximum insert size of 101 aa. This study reflected the need for incorporating flanking regions with the epitopes used in this study of the VP7 vaccine delivery system. This may not be true for all epitopes. Previous studies using only HIV epitopes failed to elicit insert specific immune responses. VP7 is capable of displaying large inserts however, depending on the site of insertion, particle assembly can be affected by decreases in the hydrophobic character of the insert but it did not affect the particles ability to display an epitope which is conformationally antigenic. We also know that limitations do exist on the hydrophilic character of the insert on site 200 with regards to particle assembly into large particles. Site 177 is therefore more tolerant of foreign peptides as
assembly into large particles remains largely unaffected. The association of VP7mu200 and the chimeras with lipid or protein raises the question whether VP7 is present as an inclusion body in insect cells. The hexagonal structure of VP7mu200 and VP7mu177 is not able to absorb conformational changes brought by these inserts. Both sites 177 and 200 display distorted particles due to weakened hydrophobic interactions and steric interference. However, once the structural distortion has occurred the structure attained with 50 aa is stable with 101 aa. In both VP7mu200 and VP7mu177 expression of two heterogeneous inserts (Rila and Ald) did not destroy particle assembly. The insertion sites 177 and 200 differ on particle assembly possibly due to conformation constraints and disturbance of hydrophobic interactions in site 200 as site 177 occurs in the hydrophilic RGD loop which is better suited to absorb these effects caused by large inserts.

It is possible that due to the size of the large particles investigated in this study, that they may not effectively elicit an immune response. The immunogenicity of the large particles formed in fractions 1-4 should be compared with smaller particles formed in fractions 5-8 including a comparison between mechanically lysed cells and detergent treated cells. Studies involving dounce homogenization to lyse cells infected with VP7mu177 chimeras to confirm decreased density of the particles due to lipid association need to be completed. Trimerization assays and solubility studies of the VP7mu200 and VP7mu177 chimeras also need to be addressed. Confirmation is required that VP7 particles may be inclusion bodies. The nature of these inclusion bodies whether it be protein or lipid based, needs to be determined. Comprehensive conformation as well as immunogenicity studies involving the insoluble particles focused on in this investigation as well as the soluble particles should be completed which include ELISA and virus neutralisation assays. All of which will increase our knowledge of these vectors and are currently underway.
CONGRESS CONTRIBUTIONS

Local Congress Participation

Oral presentation
REFERENCES


Appendix A

Figure 1: ClustalX (1.81) Nucleotide and amino acid sequence alignment of mu200-R (pFb7mu200-R) with mu200 (pFb7mu200).

mu200        GTTTAAATTCGGTTAGGATGGACGCGATACGAGCAAGAGCCTTGTCCGTTGTACGGGCAT 60
              M   D  A  I  R  A  R  A  L  S  V  R  C
mu200-R      GTTTAAATTCGGTTAGGATGGACGCGATACGAGCAAGAGCCTTGTCCGTTGTACGGGCAT 60
              M   D  A  I  R  A  R  A  L  S  V  R  C

mu200        GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA 120
              V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I
mu200-R      GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA 120
              V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I

mu200        TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC 180
              A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q
mu200-R      TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC 180
              A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q

mu200        AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG 240
              A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V
mu200-R      AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG 240
              A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V

mu200        TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG 300
              Q  I  G  N  I  S  P  D  Y  D  Q  A  L  V  G  A  L  A 95
mu200-R      TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG 300
              Q  I  G  N  I  S  P  D  Y  D  Q  A  L  V  G  A  L  A 95

mu200        CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC 360
              T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q
mu200-R      CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC 360
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mu200        AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG 420
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mu200-R      AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG 420
              M  Q  T  F  G  P  S  K  V  Q  A  M  N  D  I  V  R  I  T  G  Q

mu200        TGCAACAATCTGGCAGATATTACGTACCGCAAGGTCGAACGCGTGGTGGGTACATCAATT 480
              Q  Q  S  G  R  Y  V  Y  P  Q  G  R  T  G  Y  I  N  S
mu200-R      TGCAACAATCTGGCAGATATTACGTACCGCAAGGTCGAACGCGTGGTGGGTACATCAATT 480
              Q  Q  S  G  R  Y  V  Y  P  Q  G  R  T  G  Y  I  N  S

mu200        CAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAG 540
              N  I  A  E  V  C  M  D  A  G  A  A  G  Q  V  N  A  L  A 175
mu200-R      CAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAG 540
              N  I  A  E  V  C  M  D  A  G  A  A  G  Q  V  N  A  L  A 175
Figure 2: ClustalX (1.81) Nucleotide and amino acid sequence alignment of mu200-A (pFb7mu200-A) with mu200 (pFb7mu200).

mu200: GTTTAAATTCGTTAGGAGCAGCGATACGAGCAAGAGCCTTGCTGTTACCGGAT
       MAIRARALSVRAC

mu200-A: GTTTAAATTCGTTAGGAGCAGCGATACGAGCAAGAGCCTTGCTGTTACCGGAT
       MAIRARALSVRAC

mu200: GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA
       VTVDARVSLDPGVMETLGI

mu200-A: GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA
       VTVDARVSLDPGVMETLGI

mu200: TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC
       AINRYNLNTSHSVSRPQT

mu200-A: TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC
       AINRYNLNTSHSVSRPQT

mu200: AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG
       AERNMFFMDVADLTVGAL

mu200-A: AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG
       AERNMFFMDVADLTVGAL

mu200: TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG
       QIGNISPDYDQALTVGAL

mu200-A: TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG
       QIGNISPDYDQALTVGAL

mu200: CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC
       TTEIPYNQAMNDIVRITG

mu200-A: CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC
       TTEIPYNQAMNDIVRITG

mu200: AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG
       MQTFGPSKVQTPYAGAVE

mu200-A: AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG
       MQTFGPSKVQTPYAGAVE

mu200: TGCAACAATCTGGCAGATATTACGTACCGCAAGGTCGAACGCGTGGTGGGTACATCAATT
       QQSRYVPQGRTRGYINS

mu200-A: TGCAACAATCTGGCAGATATTACGTACCGCAAGGTCGAACGCGTGGTGGGTACATCAATT
       QQSRYVPQGRTRGYINS

mu200: CAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAG
       NIAEVMDAGAAQGVNALLA

mu200-A: CAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAG
       NIAEVMDAGAAQGVNALLA

mu200: CCCCAAGGAGGGGGAGCGACTGATGATGATGAGGAGGTTGCGCTGCTAG
       PRGDADVIMYFVWRPLRFC

mu200-A: CCCCAAGGAGGGGGAGCGACTGATGATGATGAGGAGGTTGCGCTGCTAG
       PRGDADVIMYFVWRPLRFC
Figure 3: ClustalX (1.81) Nucleotide and amino acid sequence alignment of mu200-RA (pFb7mu200-RA) with mu200 (pFb7mu200).

mu200

```
M D A I R A R A L S V V R A C 15
```

mu200-RA

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M D A I R A R A L S V V R A C 15
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V T V T D A R V S L D P G V M E T L G I 35
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A I N R Y N G L T N H S V S M R P Q T 55
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A I N R Y N G L T N H S V S M R P Q T 55
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Q I G N I S P D Y D Q A L A T V G A L A 95
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Q I G N I S P D Y D Q A L A T V G A L A 95
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A E R N E M F F M C T D M V L A A L N V 75
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A E R N E M F F M C T D M V L A A L N V 75
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Q I G N I S P D Y D Q A L A T V G A L A 95
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Q I G N I S P D Y D Q A L A T V G A L A 95
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M Q T F G P S K V Q T G P Y A G A V E 135
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Q Q S G R Y Y V P Q G R T R G Y I N S 155
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Q Q S G R Y Y V P Q G R T R G Y I N S 155
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N I A E V C M D A A G A G Q V N A L L A 175
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N I A E V C M D A A G A G Q V N A L L A 175
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