The development of vaccine delivery systems based on presenting peptides on the surface of core protein VP7 of African horse sickness virus

by

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SUMMARY

The development of vaccine delivery systems based on presenting peptides on the surface of core protein VP7 of African horse sickness virus

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Novel vaccine strategies for the presentation of immunologically important epitopes to the immune system are continuously being developed. Two such systems include the particulate protein and live viral vector delivery systems. In his study the long-term objective is to explore the African horsesickness virus (AHSV) serotype 9 viral protein 7 (VP7) and the Lumpy skin disease (LSDV) viral vector as two different vaccine strategies, particularly in view of the development of an HIV-1 vaccine. Consequently two very specific objectives were outlined in this study. The first was to express the HIV-1 subtype C strain Du 151 gp41 epitopes ALDSWK and RVLAIERYLKD on the surface of the AHSV-9 VP7 particulate protein crystalline structures. A longer-term aim is to synthesise large quantities of these chimeric VP7 crystals in order to assess the immune response against the inserted epitopes. Secondly, the efficiency of the LSDV bi-directional promoter pA7LA8R in expressing chimeric VP7 proteins was to be evaluated by utilising the late element of this promoter to determine expression levels.

Nucleotide sequences encoding the ALDSWK and RVLAIERYLKD epitopes were amplified from the HIV-1 subtype C strain Du 151 gp160 gene utilising PCR. These sequences were cloned individually as well as in combination into a multiple cloning site (549-566bp) present in the AHSV-9 VP7 gene. Recombinant pFASTBAC vectors PFASTBAC-VP7-MT 177-RVLAIERYLKD, PFASTBAC-VP7-MT 177-ALDSWK AND PFASTBAC-VP7-MT-177-RVLAIERYLKD-ALDSWK were identified, sequenced

and used in the generation of recombinant baculoviruses utilising the BAC-to-BAC™ Baculovirus expression system. Expression of all three chimeric proteins, VP7-ALDSWK, VP7-RVLAIERYLKD and VP7- RVLAIERYLKD-ALDSWK was detected in infected Sf9 insect cells utilising SDS-PAGE. Further investigations will involve high-level expression of these proteins, which in turn will allow their characterisation as well as solubility, scanning electron and immunogenicity studies.

In order to evaluate the efficiency of the LSDV bi-directional promoter, the AHSV-9 VP7 gene was cloned under the control of the late element (pA7L) of this promoter. The recombinant pHSsgpt-VP7 transfer vector was subsequently transfected into lamb testis cells infected with wild type LSDV in order to generate recombinant LSDV-VP7. Several rounds of recombinant virus selection in the presence of mycophenolic acid resulted in the loss of the LSDV-VP7 recombinant. Due to this unforeseen result, the expression of the VP7 protein from the late element of the pA7LA8R bi-directional promoter could not be quantified and the efficiency of this promoter was not determined. The loss of LSDV recombinants, which contain a gene under the control of the late promoter element pA7L, has occurred previously and is suspected to be because of the instability of these recombinants.

Due to the difficulties inherent in working with the LSDV viral vector delivery system, it has subsequently been decided to explore an alternate poxviral vector system. The focus in this study is now being shifted onto the promising Modified Vaccinia Ankara (MVA) viral vector system.

ABBREVIATIONS

Ab	antibody
AcNPV	*Autographa californica* nuclear polyhedrosis virus
AHSV-9	African horsesickness virus serotype 9
Amp	ampicillin
ATP	adenosine triphosphate
ATV	activated trypsin versene
bp	base pairs
BSA	bovine serum albumin
BTV	bluetongue virus
β-gal	β-galactosidase
β-ME	β-mercaptoethanol
$CaCl_2$	calcium chloride
CO_2	carbon dioxide
CPE	cytopathic effect
CPV	canarypox virus
CsCl	cesium chloride
CTL	cytotoxic T lymphocytes
°C	degrees Celsius
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dUTP	2'-deoxyuracil-5'-triphosphate

ddH_20	double distilled water
DEPC	diethylpyrocarbonate
dH_20	distilled water
DIG	digoxigenin
DMEM	Dubelco's Modified Eagles Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	2'-deoxy-nucleotide-5'-triphosphate
ds	double stranded
dsDNA	double stranded deoxyribonucleic acid
DTT	1,4-dithiothreitol
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
e.g.	exempli gratia (for example)
et al.	et alia (and others)
EtBr	ethidium bromide
EtOH	ethanol
FBT	fetal bovine testis
FCS	fetal calf serum
Fig.	figure
FPV	fowlpox virus
gpt	*E. coli* xanthine-guanine phosphoribosyl transferase
HAT	hypoxanthine, aminopterin and thymidine

HCl	hydrochloric acid
H_20	water
hr	hour
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
kDa	kiloDalton
KLH	keyhole limpet hemocyanin carrier protein
λII	lambda DNA molecular weight marker II
LB broth	Luria-Bertani broth
LiCl	lithium chloride
LSDV	lumpy skin disease virus
LT	lamb testis
M	molar
mA	milliampere
MCS	multiple cloning site
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	millilitre
mM	millimolar
M.O.I.	multiplicity of infection
MPA	mycophenolic acid
Mr	molecular weight
mRNA	messenger ribonucleic acid
$MgSO_4$	magnesium sulphate

m/v	mass per volume
MVA	modified vaccinia Ankara
NaCl	sodium chloride
NaI	sodium iodide
NaOAc	sodium acetate
NaOH	sodium hydroxide
ng	nanogram
NIV	National Institute of Virology
nm	nanometer
OD	optical density
O/N	overnight
OVI	Onderstepoort Veterinary Institute
ϕX	PhiX174/*Hae* marker
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-A	phosphate buffered saline without calcium and magnesium, containing 0.2% gentamicin
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
p.i	post infection
pmol	picomol
PSB	protein solvent buffer
r.e.	restriction endonuclease
RNA	ribonucleic acid

rpm	revolutions per minute
RT	room temperature
SA	South Africa
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second
Sf9	*Spodoptera frugiperda*
SPV	swinepox virus
ss	single stranded
TAE	tris acetate EDTA
Taq	*thermus aquaticus*
TE	tris EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
tet	tetracycline hydrochloride
TGS	tris glysine sodium dodecyl sulphate buffer
TK	thymidine kinase
Tris	tris-(hydroximethyl)-aminomethane
U	units
µg	microgram
µl	microliter
USA	United States of America
UV	ultraviolet
V	volts
VP	viral protein

v/v	volume per volume
WHO	world health organisation
wt	wild type
w/v	weight per volume ratio
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Since the introduction of the concept of vaccination by Edward Jenner in the late 1700's, a number of effective animal and human vaccines have been developed. Most notable of these has been the vaccinia virus vaccine that was used in an extensive vaccination program and resulted in the global eradication of the deadly human disease smallpox. This was accomplished in 1977 and certified in 1979 by the World Health Organisation.

The term 'traditional' vaccines encompasses all vaccines developed prior to 1980. These vaccines include both "live" and "inactivated" vaccines. Live vaccines are traditionally composed of attenuated viruses/bacteria that have been selected for their reduced pathogenicity while inactivated vaccines consist of inactivated, whole pathogens. Although these traditional vaccines have played an important role in the control of disease, safety concerns regarding their use have arisen. Reversion of attenuated viral vaccine strains back to their virulent phenotype via mutations or recombination with virulent strains is always a risk (Sheppard, 1999). As is the use of inactivated viral vaccines where the inactivation of virulent pathogens is not always one hundred percent effective. These concerns have resulted in the development of a new generation of recombinant vaccines (Ellis, 1999).

Utilising recombinant DNA biotechnology, recombinant vectors, capable of expressing heterologous foreign proteins, protein fragments or small peptides, have been constructed. When presented in their optimal immunological conformation to the immune system by these vectors, the peptides are able to elicit an effective immune response against a particular pathogen. In addition, the absence of the pathogens entire genome in such vaccines ensures that no infectious particles can be synthesised and consequently no disease manifests. The various recombinant vaccine systems include, amongst others, the particulate protein and live viral vector delivery systems.

Although the generation of an immune response against foreign proteins or large fragments of foreign proteins is easily achieved, the induction of such a response against small synthetic peptides or epitopes is, however, notoriously difficult. Small peptides and epitopes have, until recently, been conjugated to larger carrier proteins such as bovine serum albumin (BSA) in order to induce an epitope-specific antibody immune response. As an alternative to the use of carrier proteins, epitopes are now being inserted into and presented on the surface of various proteins, including the hepatitis B core protein (HBc) and the African horse sickness viral protein 7 (VP7). Such particulate vaccine strategies are able to induce an antibody-mediated (humoral) immune response against a specific pathogen.

Although most vaccine strategies are heavily dependent on antibodies that target extracellular pathogens in the blood and lymph in the case of intracellular pathogens, such as HIV, antibodies alone will not provide adequate protection against disease. In recent years, the induction of a cellular immune response against intracellular pathogens has received much attention. Currently, one of the best strategies for the induction of a sufficiently strong cellular immune response is the live viral vector delivery system (Hanke *et al.*, 1999; Liljeqvist & Stahl, 1999). Among the most promising viral vectors are members of the Poxviridae family including modified vaccinia Ankara (MVA), canarypox virus (CPV), fowlpox virus (FPV), swinepox virus (SPV) and lumpy skin disease virus (LSDV). Co-incidentally certain recombinant poxviral vectors, such as vaccinia, have been found to induce high titres of neutralising antibodies against both the target pathogen and poxviral vector (Berinstein *et al.*, 2000; Kutinova *et al.*, 2001). It has been shown that recombinant vectors, such as the modified vaccinia Ankara (MVA) vector, are also able to induce neutralising antibodies in vaccinated individuals, albeit at lower titres than vaccinia virus (Zhu *et al.*, 2000). Upon virulent viral challenge, individuals vaccinated with MVA recombinants have been found to elicit high levels of neutralising antibodies against the target pathogen. MVA may thus be used to prime individuals for an anamenestic neutralising antibody response (Ourmanov *et al.*, 2000; Barouch *et al.*, 2001).

In this study, both these strategies are to be explored. Epitopes will be presented to the immune system on the surface of VP7 particulate protein structures and will also be delivered directly into target cells via a recombinant poxviral vector, expressing the same

chimeric VP7 construct. It will thus be possible to determine which system is able to induce the greatest titre of neutralising antibodies.

As the acquired immunodeficiency syndrome (AIDS) pandemic continues to spread, researchers are urgently seeking a vaccine against the causative agent the human immunodeficiency virus (HIV). Candidate vaccines, such as the most recent HIV recombinant glycoprotein 120 (rgp120) subunit vaccine, have been unsuccessful in protecting individuals against HIV infection (Conner *et al.*, 1998). A recent novel approach in HIV vaccine design was demonstrated by Chen *et al.*, 1999a. By utilising so-called 'epitope' or 'multiepitope' vaccines, these authors succeeded in targeting and enhancing the humoral immune response to a few strong neutralisation-specific HIV epitopes. As HIV is an intracellular pathogen, the presence of neutralisation antibodies will not suffice to protect against the progression of disease post-infection. However, the combination of a particulate protein and a live viral vector vaccine systems in a prime-boost strategy may be sufficient to induce high levels of both humoral and cellular immunity against HIV.

In this literature review, the use of the particulate (AHSV) viral protein 7 system for the presentation of immunologically important epitopes to the immune system will be motivated as will the use of a live LSDV poxviral vector system for the delivery of the chimeric VP7 protein into target cells. A prime boost strategy, which involves priming the immune system with a recombinant viral vector and subsequently boosting this response with a homologous protein, will be also reviewed. The suitability of above-mentioned systems as vaccine delivery systems will be determined following the insertion of specific HIV-1 epitopes into these vectors. The motivation behind the use of these specific HIV-1 epitopes will be discussed and will include a broad overview of HIV, current HIV vaccines as well as the novel HIV-1 'epitope vaccine' approach.

1.2 A PARTICULATE AHSV-9 VIRAL PROTEIN 7 PRESENTATION SYSTEM

In order to induce an antibody-mediated immune response against a specific small peptide, the peptide can be presented on the outer surface of a carrier structure (particle) built in a symmetric manner from hundreds of proteins of one or more types. As such structures ensure that the peptide attains its correct three-dimensional

conformation and contain a high density of particles per peptide, they would ensure the induction of a good humoral immune response against the presented peptide (Pumpens & Grens, 1999). Such particulate protein carriers include, amongst others, the hepatitis B core antigen, HbcAg (Pumpens & Grens, 1999), which has recently been used in the development of a vaccine against malaria (Milich *et al.*, 2002) as well as the non-structural protein NS1 of Bluetongue virus, BTV (Mikhailov *et al.*, 1996). Epitopes could also be presented to the immune system on a scaffold of the African horse-sickness virus (AHSV) viral protein 7 (VP7) crystals. First suggested by Dr. Wade-Evans (personal communication), this novel idea is based on the unique characteristics of the AHSV VP7 protein.

As reviewed by Roy *et al.,* (1994), African horse-sickness (AHS) is a disease that is endemic to Sub-Saharan Africa and results in a high mortality rate among horses. The causative agent, African horse-sickness virus, is classified as a member of the genus Orbivirus in the family Reoviridae. Nine different serotypes of AHSV have to date been identified in Africa, AHSV-1 to AHSV-9 (McIntosh, 1958; Howell, 1962).

The first molecular characterisation of AHSV was performed by Verwoerd and Huismans (1969). The morphology and molecular constituents of AHSV are essentially identical to those of bluetongue virus (BTV), the prototype Orbivirus. AHS viral particles are composed of seven structural proteins, VP1 - VP7, that assemble to form a double capsid. The outer capsid of the mature virion consists of two proteins, VP2 and VP5, while the inner capsid or core consists of the major proteins VP7 and VP3 as well as three minor proteins VP1, VP4 and VP6. VP7 is the main component of the AHSV inner core particle (Roy *et al.,* 1994). X-ray diffraction studies of BTV VP7 crystals expressed by recombinant baculoviruses indicate that the protein is grouped together in trimeric structures (Basak *et al.,* 1992). The VP3 protein in the core serves as a scaffold for these clusters of VP7 trimers (Roy, 1992).

The core encloses the viral genome, which consists of ten double stranded (ds) RNA segments of varying sizes (Roy, 1992). One of the smaller ds RNA segments S7 encodes the AHSV VP7 polypeptide. The 349 amino acid protein, with a calculated Mr of 38 kDa, is conserved between all three culicoides (biting midges)-transmitted orbiviruses namely, AHSV, BTV and epizootic haemorrhagic disease virus, EHDV (Roy *et al.*, 1991;

Roy *et al.*, 1994). The amino acid sequence of VP7 between various AHSV serotypes is more highly conserved, with AHSV-4 and ASHV-9 exhibiting 99.7% amino acid identity (Roy *et al.*, 1994).

AHSV VP7 is composed of 349 amino acids with a calculated Mr of 38 kDa and is an extremely hydrophobic protein (Roy *et al.,* 1991). It was first documented by Chuma *et al.,* (1992) that high levels of AHSV-4 VP7 trimers, expressed by a recombinant baculovirus, were able to self-assemble to form disc-shaped crystal structures in baculovirus-infected insect cells. These flat, hexagonal VP7 crystals were subsequently observed in AHSV-9 infected BHK-21 cells with diameters ranging from 2-5μm (Burroughs *et al.,* 1994). These crystals have a highly ordered hexagonal lattice consistent with a trimeric subunit structure. This hexagonal arrangement appears to have a direct structural similarity to the segmented, ring-shaped capsomers that are visible on the outer surface of the AHSV core (Burroughs *et al.,* 1994). The formation of such crystalline structures appears, to date, to be a characteristic unique to AHSV VP7, not even the closely related BTV has the ability to form such crystals.

AHSV VP7 crystals, purified from baby hamster kidney cells infected with AHSV-9, were shown to be highly immunogenic and effective subunit vaccines against AHSV in the mouse model (Wade-Evans *et al.*, 1997; Wade-Evans *et al.*, 1998). However the protection observed was not due to an induced neutralising antibody response as the passive transfer of antibodies from immunised mice failed to protect recipients from virulent AHSV challenge. The induction of a T cell mediated immune response by the VP7 protein was reasoned to be the primary mechanism responsible for the protection observed. Subsequent reports that the VP7 protein of BTV contains immunodominant T-cell epitopes (Angove *et al.*, 1998 as cited by Maree, 2000) prompted Dr Wade-Evans to suggest that VP7 be investigated as a particulate antigen delivery system for the presentation of epitopes to the immune system.

The AHSV VP7 monomer is composed of two distinct domains. A top domain, composed of amino acids 121-249, that is folded into an anti-parallel β sandwich and a lower domain containing the first 120 amino acids at the N-terminus and the last 99 residues at the C-terminus (Basak *et al.,* 1996). X-ray crystallography was used to elucidate that during crystallisation, the two-domain VP7 protein is cleaved with the result that only the

top domain remains (Basak *et al.,* 1996). If these VP7 crystals are to be evaluated as a system to present immunologically important epitopes to the immune system, the epitopes should be inserted into the top domain of VP7. The presence of at least five hydrophilic regions in the top domain of VP7, exposed on the surface of the VP7 crystalline particles, present the possibility of these regions being used for the insertion and presentation of epitopes. A particularly attractive target for the insertion of epitopes is the arginine-glysine-aspartic acid (RGD) motif, located on a highly flexible loop (aa 175-180) in the top domain of VP7. This motif is used for the attachment of the AHSV core particles to the cell membrane. To allow for the insertion of epitopes into the RGD motif, a multiple cloning site encoding six extra amino acids was inserted between the amino acids 177-178 of AHSV-9 VP7 (Maree, 2000). The insertion of the six additional amino acids in this position resulted in the increase a six-fold increase in VP7 solubility and a change in the shape from flat crystals to more rounded tennis-ball structures (Maree, 2000). This observation could prove advantageous to the design of a vaccine as the surface area available for the presentation of the epitopes is increased.

1.3 A LIVE LUMPY SKIN DISEASE POXVIRAL DELIVERY SYSTEM

As antibodies primarily target extracellular pathogens in the blood and lymph, they are unlikely to play a major role in the control of intracellular pathogens (Burton, 1997). Once infection has taken place, the intracellular replication and cell-to-cell spread of the pathogen can only be contained by the destruction of infected host cells (Mascola & Nabel, 2001). CD8+ cytotoxic T lymphocytes (CTL's) are responsible for the destruction of these infected host cells (reviewed in Whitton & Oldstone, 1996). The advantage of utilising live viral vectors for the delivery of foreign heterologous proteins into target cells lies in the their ability to induce a CTL response against a specified pathogen (Hanke *et al*., 1999; Liljeqvist & Stahl, 1999). This is due to the virus's inherent ability to infect and replicate within target cells and the presence of appropriate CTL epitopes in the expressed heterologous protein.

Among various recombinant viral vectors, poxviruses have been the most widely studied (Mascola & Nabel, 2001). Among the properties that make them desirable for use as recombinant viral vaccines is their ability to grow to high titres (10^{10} pfu/ml), their ability to retain their infectivity and their high thermostable ability (Tartaglia *et al*, 1990).

Poxviruses also have a large capacity for the insertion of foreign DNA, which makes it possible to construct polyvalent vaccines, which simultaneously express multiple antigens (Flexner *et al.,* 1988 as quoted by Durbin *et al.,* 1998).

In 1982, the first foreign genes were introduced into the Orthopoxvirus vaccinia virus (Mackett *et al.,* 1982; Panicali & Paoletti, 1982 as quoted by Moss, 1996). Vaccinia virus was first used in the vaccination campaign that resulted in the eradication of smallpox in 1979. An attenuated vaccinia that expressed the HIV gp160 envelope protein was the first recombinant vector evaluated in humans (Cooney *et al.*, 1991; Cooney *et al.*, 1993). However, safety concerns about vaccinia vector arose when it was found that immunocompromised individuals experienced complications after inoculation with the smallpox virus vaccine (Redfield *et al.,* 1987; Fenner, 1988, both as quoted by Moss, 1996). Another concern was that the wide-host range of vaccinia might lead to the spread of the virus to non-target species (Brown, Schild & Ada, 1986). Due to the large numbers of immuno-compromised individuals world-wide that are a direct consequence of the global AIDS epidemic, alternate poxviruses are now being considered as vectors for live viral vaccine applications. These include highly attenuated vaccinia vectors for example modified vaccinia Ankara (MVA) or the use of species-specific poxviruses, both of which are unable to replicate and cause disease in humans.

MVA is a severely attenuated and host-range restricted vaccinia virus strain (Moss, 1996). This promising viral vector is non-virulent in immunosuppressed people (Wyatt *et al.,* 1998) and a number of MVA-recombinants have been used to immunise successfully against several diseases by eliciting a strong CTL response (Sutter *et al.,* 1994; Wyatt *et al.,* 1996). Indeed, the MVA vector has been said to be to be as or more effective than replication competent vaccinia as a recombinant viral vector (Sutter *et al.*, 1994; Hirsch *et al.*, 1996). Recent studies have found that macaques, immunised with recombinant MVA-SIV vaccines and challenged with pathogenic strains of SIV, showed lower viral loads and prolonged survival relative to control animals that received non-recombinant MVA (Seth *et al.*, 2000; Ourmanov, Brown *et al.*, 2000; Barouch *et al.*, 2001). The magnitude of reduction in viremia in vaccinees was inversely correlated with the magnitude of vaccine-elicited CTL response prior to SIV challenge (Seth *et al.*, 2000; Barouch *et al.*, 2001). The above results demonstrate that although live recombinant vectors, such as MVA, are unable to provide sterilising immunity, they are capable of

controlling viremia and preventing disease progression, following a highly pathogenic AIDS virus challenge. A MVA vector, expressing the HIV-1 gag gene and a number of CTL epitopes, has recently entered clinical trials in the UK and Kenya (cited by Johnston & Flores, 2001).

Species-specific poxviruses, also candidate vectors for live viral vaccines, have host ranges that are restricted to particular animal groups. Although these poxviruses can efficiently infect mammalian cells, complete viral replication does not occur and infectious virus is not produced. Despite their incomplete replication cycle, these recombinant poxviruses have the ability to express foreign genes and the foreign proteins elicit a protective immune response in mammals (Taylor *et al.*, 1988). Potential vectors include the avian poxviruses fowlpox virus (McMillen *et al.,* 1994 as cited by Yamanouchi *et al.*, 1998) and canarypox virus (Taylor *et al.,*1995), swinepox virus (van der Leek *et al.*, 1994; Paoletti, 1996; Tripathy, 1999) and lumpy skin disease virus (LSDV) (Ngichabe *et al.,* 1997).

Recombinant fowlpox (FPV) and canarypox (CPV) viruses have previously been employed as veterinary vaccine vectors (Webster *et al.*, 1991; Boursnell *et al.*, 1990; Bayliss *et al.*, 1991; Taylor *et al.*, 1991). In the past few years, however, these vectors have been utilised in the design of an HIV-1 vaccine. Studies show that FPV and CPV recombinants expressing the HIV-1 Env gene are capable of eliciting humoral and T-cell immune responses in rabbits that inhibit specific HIV-1 functions (Radaelli *et al.*, 2000). In a combined DNA-prime FPV boost strategy, several FPV-HIV recombinants were found to significantly increase the cellular responses to and protection against HIV-1 in macaques (Kent *et al.*, 1998). However, the canarypox virus is able to induce superior levels of protective immunity when compared to fowlpox virus (Taylor *et al.*, 1991; Taylor *et al* 1992; Radaelli *et al.*, 2000). Recent studies have shown that a recombinant canarypox vector, expressing various HIV antigens, is able to elicit CTL and T-cell memory responses in HIV-1 uninfected volunteers (Gorse *et al.*, 2001). ALVAC™, a recombinant canarypox virus developed by Aventis Pasteur, is currently the most extensively studied vector in HIV human trials (Johnston & Flores, 2001).

Swinepox virus (SPV) is another host-range restricted viral vector was used in a vaccine strategy to protect pigs against pseudorabies (van der Leek *et al.*, 1994). Some data,

however, suggests that despite the strict host tropism of SPV, the virus exhibits a relatively broad host range in cell culture (Barcena & Blasco, 1998). This could limit the further use of SPV as a recombinant viral vector.

The host-range restricted poxvirus to be evaluated as a live viral vector system in this study is Lumpy skin disease virus (LSDV). LSDV is a member of the *Capripoxviridae*, whose host range is restricted to ungulates such as sheep, goats and cattle (Matthews, 1982 as cited by Fick, 1998). The diseases that LSDV causes in these animal groups are sheeppox, goatpox and lumpy skin disease in cattle. The sheeppox virus has already been used as a recombinant vector and protects cattle against clinical rinderpest (Romero *et al.,* 1993) as well as goats against peste des petits (Romero *et al.,* 1995). Sheep were also partially protected against bluetongue virus when immunised with a recombinant sheep-pox vector (Wade-Evans *et al.,* 1996). As sheep-pox, as well as goatpox, is not endemic to South Africa, an attenuated stain of LSDV (Neethling strain), that only infects cattle and has been used for vaccine purposes since the 1960s, is a candidate that could be developed as a recombinant vector for South Africa.

1.4 A PRIME-BOOST STRATEGY

Various authors have highlighted the need for combining strong cell-mediated and neutralising antibody responses as prerequisites for effective vaccines against intracellular pathogens (Poignard *et al.*, 1996; Burton, 1997). Poxviral vectors, although able to elicit modest levels of $CD4^+$ and $CD8^+$-mediated immune response, are unable to induce high levels of anti-envelope neutralising antibodies (Mascola & Nabel, 2001). The prime boost strategy is a method by which high levels of both cellular and humoral immunity can be achieved (Corey *et al.*, 1997; Girard, 2000; Williamson *et al.*, 2000). This immunisation strategy involves priming the immune system with a recombinant poxviral vector and subsequently boosting the immune response with homologous protein.

In HIV-1 phase-1 clinical trials, the humoral and CTL immune responses induced by a vaccinia-gp160 recombinant were significantly enhanced following a boosting with the homologous gp160 protein (Cooney *et al.*, 1993). Similarly, early studies in the SIV-macaque model indicated that a recombinant vaccinia-Env virus, boosted by the soluble

envelope protein, could completely protect against a homologous pathogenic SIV challenge (Hu *et al.*, 1992). It has subsequently been revealed that complete protection is only possible in the setting of homologous viral challenge and strain-specific antibodies are likely to play a role in this protection (Polacino *et al.*, 1999). A recent phase II study involving canarypox-HIV recombinants found that even though CTL responses were only detected in 33-50% of all volunteers, high levels of neutralising antibodies and Th responses were detected in 94% of all volunteers after boosting with gp120 (Belshe *et al.*, 2001; The AIDS vaccine evaluation group 022 protocol team, 2001).

Should the particulate AHSV-9 VP7 presentation and the LSDV viral delivery systems in this study be found capable of inducing humoral and cellular immune responses, respectively, a prime-boost would be advisable. The combination of these two vaccine delivery systems in a prime boost strategy could induce humoral and cellular responses against a pathogen significantly higher than either of the two strategies individually. The immune system would firstly be primed with a recombinant Lumpy Skin Disease virus. The presence of suitable neutralisation and CTL epitopes within the VP7 protein would prime the humoral immune response and induce a cellular response against the particular pathogen. Injecting a homologous chimeric VP7 protein into previously immunised individuals would serve to boost the humoral response and achieve high levels of neutralising antibodies.

1.5. HIV- AN OVERVIEW

The human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS), has had a significant impact on the world's population in the last two decades. Although only first identified in 1983, the virus has spread rapidly, particularly in developing countries where the number of infected individuals is reaching pandemic proportions. According to the United Nations (UN) AIDS figures for 1999, an estimated 30.6 million people world-wide were living with HIV-1 while a cumulative total of 12 million had already died from AIDS related causes (International AIDS Vaccine Initiative, 1999).

The role that HIV plays in the development of the acquired immunodeficiency syndrome is well known (Ahmed *et al.*, 1996). A crucial subset of immune cells called the $CD4^+$ or T-helper cells are the main targets for the virus. During a typical course of infection these cells, which play a central role in immune signalling, are systematically disabled and killed. The result is the gradual deterioration of immune function, which at the end stage of infection, is responsible for the development of opportunistic infections and cancers that typify AIDS.

The lack of effective immune control of HIV in infected individuals has been attributed to not only the depletion of the important Th subset of immune cells but also the failure of the HIV envelope glycoprotein to elicit potent neutralising antibody responses and the ability of HIV to mutate rapidly and escape the cellular immune response (reviewed by Mascola & Nabel, 2001).

1.5.1 HIV CLASSIFICATION, VIRION STRUCTURE AND VIRAL ENTRY INTO HOST CELLS

1.5.1.1 HIV classification

The human immunodeficiency virus (HIV) is classified as a Retrovirus in the genus *Lentiviridae* (Levy *et al.*, 1994). Retroviruses are RNA viruses that contain the enzyme reverse transcriptase (RT) within their nucleocapsid. During infection of the host cell, this viral RNA-dependant DNA polymerase is responsible for the conversion of the HIV RNA genome into cDNA; a reversal of the usual genetic process. The cDNA copy is then inserted into the host cell genome by the viral enzyme integrase and becomes known as a provirus.

Due to the high infidelity of HIV RT many errors are incorporated into the viral genome during replication, resulting in a highly heterogeneous lentivirus group (Myers *et al.*, 1994 as cited by Purtscher *et al.*, 1996). These mutations occur primarily in the envelope and regulatory genes tet, rev and nef and play an important role in the escape of HIV from immune detection. Two types of HIV virus, HIV-1 and HIV-2, are distinguished based on a 55% difference in their genomic nucleotide sequence. HIV-1 is

more readily transmissible than HIV-2 and is the causative agent of the pandemic currently affecting populations worldwide.

HIV-1 is further classified into different clades or subtypes based on phylogenetic comparisons of its envelope coding sequences. A difference of 30% in nucleotide sequence between subtypes is observed (Wills *et al.,* 1996). The subtypes are sorted into three groups. The major or M group comprises subtypes A-H as well as J and K, an unknown number of subtypes belong to the outlier (O) group and the New (N) group contains subtypes belonging to neither the M or O groups. The various HIV subtypes are generally, although not exclusively, found in specific geographic regions (UNAIDS & WHO, Geneva, 1998; HIV-1 subtype report by European Commission & Joint UN programme on HIV/AIDS, 1997). HIV-subtype B predominates in North America and Europe while subtype C is the cause of AIDS epidemics in India, China and Sub-Saharan Africa.

1.5.1.2 Virion Outer capsid structure and viral entry in host cells

HIV has a diameter of 1/ 10 000 of a mm and is spherical in shape (Levy *et al.*, 1994). The outer coat of the virus, the viral envelope, is composed of a lipid bi-layer and originates from the cell membrane of an infected host cell, from which newly formed virus particles bud. Embedded in this viral envelope are 72 copies of a complex HIV protein that protrudes from its surface. This protein, known as env, is an oligomeric structure that consists of three gp120-gp41 heterodimers (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997). Each heterodimer consists of a single gp120 mature surface glycoprotein noncovalently associated with the transmembrane glycoprotein gp41. The two glycoproteins are yielded by the cleavage of a single polypeptide precursor molecule, gp160, which is encoded by the env gene (McCune *et al.*, 1988). Gp120 and gp41 have important roles in the processes of viral attachment and entry into host cells, respectively.

Gp120, as mentioned before, is a heavily glycosylated (40-50%) protein. Five relatively conserved (C1-C5) and five variable (V1-V5) regions exist within this protein (reviewed in Burton, 1997). The regions V1-V4 are each bracketed by cysteine amino acids, which form disulphide bridges, resulting in the formation of variable loops. The gp120 molecule

has an affinity for the $CD4^+$ molecule on the surface of the target cell and this interaction, as well as an additional interaction with a co-receptor, is responsible for the initial binding of HIV to T and B lymphocytes and monocytes (Trkola *et al.,* 1996; Wu *et al.,* 1996; D'Souza *et al.,* 1997; Moore & Trkola, 1997).

Recent studies reveal that gp41 plays an important role in the entry of HIV into its target cells. The extra-membrane structure of HIV gp41 can be divided into four regions: a fusion domain at the amino terminus (aa 514-540), the N-domain (aa 542-589) followed by a hydrophilic immunodominant region (aa 592-623) and a C-domain (aa 625-657) at the carboxyl terminus (Weissenhorn, 1997; Chan *et al.,* 1997). These four regions are visualised in Figure 1.1 (Dong *et al.,* 2001).

Crystallographic analysis indicates that binding of gp120 to both CD4 and a chemokine co-receptor induces conformational changes in gp41 (Chan & Kim, 1998). A fusion-intermediate is subsequently formed in which the N and C domains are transiently exposed (Weissenhorn *et al.,* 1997). This is followed by the fusion of viral and cell membranes and entry of the HIV capsid into the cell by the process termed 'receptor-mediated activation of fusion' (Poignard *et al.,* 1996).

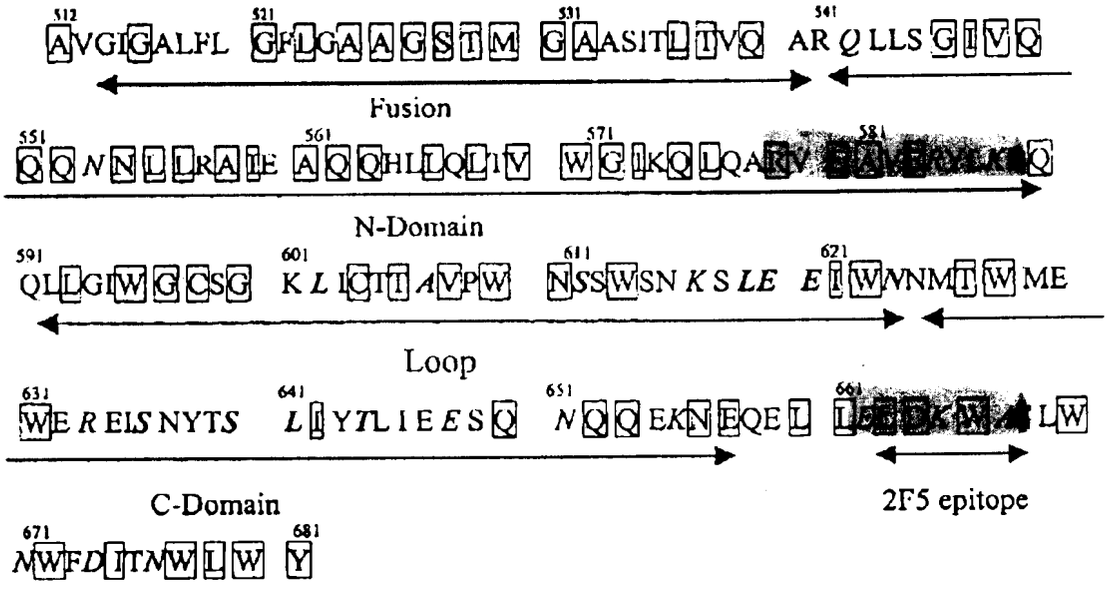

Fig 1.1 The consensus sequence of the HIV-1 gp 41 ectodomain (aa 512-681). Residues at each position throughout the ectodomain are divided into four types, according to how conserved they are. Conservative residues (over 97% conservation) are visualised in blocks. Residues that are 90-97% conserved are not marked while variable residues (50-89% conservation) are tilted. Highly variable residues that are less than 50% conserved are tilted and in bold. The arrows indicate the functional domains and highlighted are the RILAVERYLKD and ALDKWA epitope sequences. This Figure was reproduced from Dong *et al.* (2001).

1.5.3 CURRENT HIV VACCINES

As the AIDS pandemic continues to spread, researchers are urgently seeking a vaccine against HIV. The induction of a strong antibody-mediated (humoral) immune response has, until recently, been the main objective of an HIV vaccine. The importance of a candidate HIV vaccine being able to elicit potent neutralising antibodies has been indicated by several studies. Igarashi *et al.*, (1999) found that free HIV-1 virions in the blood could be bound and inactivated principally by neutralising antibodies. In addition, passive immunisation experiments, during which cocktails of antibodies were passively transferred to macaques, were found to protect the monkeys against pathogenic viral strain challenge - protection correlated with *in vitro* neutralisation results (Mascola *et al.*, 1999; Baba *et al.*, 2000). These studies lend credibility to the notion that neutralising antibodies can contribute to the control of viremia and would be an important component of an HIV-1 vaccine (Krause *et al.*, 1997; Montefiori & Evans, 1999).

As the HIV envelope is the predominant target of neutralising antibodies in HIV-infected individuals, attempts at the generation of an HIV-1 vaccine have mainly focused on immunisation with recombinant HIV envelope proteins (Johnston & Flores, 2001). Candidate vaccines, including the most recent HIV recombinant glycoprotein 120 (rgp120) subunit vaccine, were unsuccessful in protecting individuals against HIV infection (Conner *et al.,* 1998). This failure is attributed to the inability of these vaccines to induce a strong neutralising humoral immune response against representative primary HIV strains (Moore, 1995; Mascola *et al.*, 1996; Moore & Burton, 1999).

Various theories have been put forward to explain the poor performance of the monomeric gp120 and gp160 subunit vaccines. Firstly, epitopes exposed to the scrutiny of the immune system on the monomeric forms of the gp120 and gp160 proteins are hidden within the structure of the mature envelope (env) oligomer (Burton, 1997). The low antigenicity of the mature env is attributed to its oligomeric nature and the extra-ordinarily high degree of glycosylation (40-50%) of the env glycoprotein gp120 (Burton, 1997). A large number of the normally hidden epitopes, when exposed, are immunodominant and capable of inducing high titres of anti-HIV-1 antibodies (Sattentau *et al.*, 1999). However, the antibodies elicited are, in fact, non-neutralising or have a restricted neutralising specificity. Immunisation with monomeric gp120 and gp160

subunit vaccines thus results in the induction of high titres of antibodies that are directed against epitopes poorly exposed on the surface of the mature envelope oligomer of the HIV virion. Accordingly, these antibodies are unable to bind to and neutralise the intact virions of primary HIV-1 isolates.

In addition, many of the HIV vaccine candidates have been based on T-cell line adapted (TCLA) isolates (Poignard *et al.,* 1996). These viruses, isolated from the tissues of HIV seropositive individuals and maintained in immortalised cell lines, are unrepresentative of the primary viral strains currently being transmitted in populations (Daar *et al.,* 1990 as cited by Poignard *et al.,* 1996). Due to their altered env glycoprotein structure, the epitopes on TCLA env glycoprotein oligomers are more accessible to antibody binding and they are, therefore, more sensitive to neutralisation than the primary isolates (Poignard *et al.,* 1996; Burton & Montefiori, 1997; Burton, 1997). It is reasonable to conclude that vaccine candidates based on TCLA isolates would raise antibodies against regions that are well exposed on the TCLA isolates but that these antibodies would be unable to bind to the mature env oligomer of primary viruses (Mascola *et al.*, 1996; Sattentau *et al.,* 1999).

Based on the above, immunisation with the mature env oligomer of either the primary or TCLA virions would seem a more promising HIV-1 vaccine. However, although the env oligomer would most likely reproduce critical features of the mature conformation, it has a low antigenicity with a small number of epitopes being exposed on its surface (Burton, 1997). These epitopes are located either in the immunodominant regions and are highly variable in sequence or are not immunogenic and are unable to raise a good humoral immune response (Sattentau *et al.*, 1999). It is unlikely that the mature env oligomer would be a successful HIV-1 vaccine candidate, capable of eliciting antibodies of sufficient affinity and concentration to a level required for the inhibition of infectivity (Burton, 1997).

1.5.4 NOVEL HIV 'EPITOPE' VACCINES

An important determinant of antibody neutralisation is antibody affinity or how strongly an antibody binds to its epitope (Burton, 1997; Sattentau *et al.,* 1999). As neutralisation of virus *in vitro* is an accurate prediction of antibody efficiency *in vivo* (Montefiori & Evans,

1999), this suggests that neutralising antibody of sufficient affinity may completely prevent HIV infection, and antibody of slightly less affinity may provide significant clinical benefit when sterilising immunity is not achieved (Montefiori & Evans, 1999). An ideal HIV vaccine against different HIV-1 clades would thus aim to induce a high titre of high affinity neutralising antibodies against a few highly conserved immunodominant epitopes well exposed on the HIV virion (Burton, 1997). These antibodies would subsequently bind strongly to the mature env oligomer on a variety of primary challenge viruses and inhibit infectivity (Sattentau *et al.*, 1999; Nyambi *et al.,* 2000).

Chen *et al.*, (1999a) succeeded in targeting and enhancing the humoral immune response against a few strong neutralisation specific epitopes by utilising the novel 'epitope' or 'multi-epitope' vaccine approach. This novel strategy involves immunisation with a synthetic peptide containing mimics of potent neutralising epitopes. The epitopes utilised in such a strategy must be linear epitopes. Linear epitopes are not dependant on their secondary and tertiary conformations but are able to elicit neutralising antibodies when presented in the form of a short peptide. Several such epitopes have been characterised for example GPGRAF (Env amino acids 316-321) on the V3 loop of gp120; ELDKWA, Env amino acids 669-674, adjacent to the C-domain of gp41 (Muster *et al.*, 1993) and RILAVERYLKD, Env amino acids 586-596, on the N-domain of gp41(Chen & Dierich, 1998). The two last-named epitopes will be discussed in detail in this literature review.

When designing a vaccine, it is important to incorporate regions from the virus that are conserved and have previously exhibited some protective activity. Two such regions in the HIV-1 virion are the N and C-domains of gp41 (Chen & Dierich, 1996; Dong *et al.,* 2001). These domains play a vital role in mediating membrane fusion and HIV-1 entry into target cells (Dong *et al.*, 2001). The N-domain is involved in the binding of the cellular receptor protein P45 (Xiao *et al.*, 2000a), while the C-domain binds to the putative cellular receptor protein P62 (Chen *et al.,* 1999b). It is clear that these domains have an important function and are highly conserved. The amino acid sequences of the gp41 ectodomain (aa 512-681) of 862 HIV-1 isolates from clades A-I, J and O were compared and analysed (Dong *et al.,* 2001). The fusion and N-domains of gp41 seemed to be highly conserved while the amino acid residues in the C-domain were less

conserved. The hydrophilic immunodominant region was the most variable region of gp41.

Evidence supporting the protective functions of these domains has also been found. Synthetic N- and C-domain peptides potently inhibit membrane fusion induced by both laboratory adapted strains and primary isolates of HIV (Jiang *et al.,* 1993; Chan *et al.,* 1997). Also, a phase I clinical trial conducted with a C-domain peptide showed significant reduction in the viral loads (Kilby *et al.,* 1998). Domains of Simian immunodeficiency virus transmembrane protein gp32 (SIV Env aa 582-602, aa 647-667), homologous to the N and C domains of gp41, were immunogenic in macaques (Shafferman *et al.,* 1991). The antibodies elicited against these regions protected the monkeys against SIV infection. In addition, LaCasse *et al.,* (1999) concluded that the effectiveness of their fusion-competent vaccine, which neutralised 23/24 primary viral isolates, was because it was a fusion intermediate formed during viral entry into the target cell. This suggests that the N and C domains, transiently exposed in the fusion intermediate, could induce protective activity. However, although the N and C-domains are strongly immunogenic when exposed on the fusion intermediates, they are generally hidden from the scrutiny of the immune response due to the oligomerisation of gp41 on the surface of virions (Dong *et al.*, 2001).

As the N and C domains of gp41 are critical to HIV entry, it seems likely that epitopes in these domains would be conserved while also being able to induce neutralising antibodies with protective activity. One such epitope is ELDKWA (Env amino acids 669-674), present adjacent to the C-domain of gp41 and highly conserved among HIV-1 subtype B isolates (Muster *et al.,* 1993; Dong *et al.,* 2001). The human monoclonal antibody (mAb) 2F5 binds to this epitope, primarily at the core amino acid sequence LDKW (Muster *et al.,* 1993; Muster *et al.,* 1994; Purtscher *et al.,* 1994; Sattentau *et al.,* 1995). Monoclonal antibody 2F5 is both potently and broadly neutralising, with the ability to neutralise a variety of both laboratory and primary viral isolates of various subtypes (Muster *et al.,* 1993; Muster *et al.,* 1994; Purtscher *et al.,* 1994; Conley *et al.,* 1994; Trkola *et al.,* 1995). Recent studies have found that 2F5, at acceptable concentrations, was able to neutralise 90% of the studied African, Asian, American and European primary isolates from clades A, B and E (Purtscher *et al.,* 1994; Purtscher *et al.,* 1996). In a complementary study, 2F5 was one of three antibodies that was capable

of neutralising a majority of typical U.S. primary isolates (D'Souza *et al.,* 1997). In addition, chimpanzees infused with 2F5 were able to delay the establishment of infection and viremia after inoculation with a primary isolate (Conley *et al.,* 1996). Although mAb 2F5 has great protective capabilities, its occurrence in infected individuals is rare (Burton, 1997). At least 80% of HIV-1 infected children do not elicit the antibody (Geffin *et al.,* 1998) and it has never been raised by a vaccine candidate (Moore & Trkola, 1997).

Another promising epitope that is able to induce protective, neutralising antibodies is RILAVERYLKD situated on the Envelope protein, amino acids 586 –596 (Chen & Dierich, 1998). A similar epitope is SIV gp32, which was found to protect macaques from SIV infection (Shafferman *et al.*, 1991). This highly conserved epitope is located in the N-domain of gp41 (Dong *et al.*, 2001) and is responsible for the binding of gp41 to the potential receptor protein P45 (Xiao *et al.,* 2000a).

Whether the ELDKWA and RILAVERYLKD epitopes are exposed on the surface of intact infectious virions of HIV-1 primary isolates is currently being debated. After testing which of the 47 human mAb directed at epitopes in gp120 and gp41 were able to bind to the surface of 26 intact, native HIV-1 isolates of various clades, Nyambi *et al.,* (2000) reported that mAb 2F5 did not bind or bound weakly. It was concluded that the ELDKWA epitope is poorly exposed on the surface of intact, infectious virions. These authors also found the RILAVERYLKD epitope to be in the cluster I region (aa 579-613) and well exposed on the virion surface. In contrast, Earl *et al.,* (1997) excluded RILAVERYLKD from the highly exposed, conserved region that they termed cluster I (597-613). In addition, they found that their recombinant, oligomeric gp140 subunit vaccine was not able to elicit antibodies to the epitope ELDKWA. This gp140 subunit vaccine contains gp120 and the ectodomain of gp41 and mimics the conformational structure of gp160. However, mAb 2F5 is able to bind to gp140. The evidence of these authors suggests that the ELDKWA epitope is exposed on the surface of intact, native HIV-1 virions but is not immunogenic.

Thus, if both epitopes are indeed exposed on the surface of intact infectious HIV-1 virions of primary isolates from various subtypes, the neutralising antibody elicited by these epitopes may exert their protective activity by binding to the virions and

subsequently changing their conformation. This action might inhibit the binding of the virion gp120 to the cellular receptor CD4. Alternatively, the neutralising antibodies may bind to the N and C-domains following viral attachment to the target cells, when their epitopes are transiently exposed in the fusion intermediate (Nyambi *et al.,* 1998). The binding of these neutralising antibodies to the N and C-domains would inhibit the interaction of these domains with their cellular receptors, P45 and P62 respectively, and thus preventing membrane fusion and entry of the HIV nucleocapsid into the host cell cytoplasm.

As the epitopes ELDKWA and RILAVERYLKD occur on the C and N domains of gp41 respectively, it would be tempting to use these domains as epitope-based peptide vaccines. However, various authors have shown that the epitope-specific antibody response induced by peptide vaccines representing these domains is not optimal. For example Xiao *et al.,* (2000b) found the ELDKWA-specific antibody response in mice to be in the order of 1: 1600-3200 when the epitope was presented in the form of the C-domain (peptide P2), conjugated to carrier peptides BSA (Bovine serum albumin) or P24EC. These antibody levels were higher than those obtained after immunisation with a rgp160 subunit vaccine (1:400), but were, nevertheless, weak and inadequate. Chen *et al.,* (1999a) drew similar conclusions about both epitopes ELDKWA and GPGRAFY when they were presented as synthetic peptides, conjugated to carrier proteins BSA or P24EC. In mice the antibody responses elicited against these epitopes were only 2-4 fold higher than those obtained after immunisation with the rgp160 subunit vaccine. Although the N and C-domains of gp41 induce a higher epitope-specific neutralising immune response than recombinant gp160 subunit vaccine, the immunogenicity of the epitopes presented on such peptide vaccines is unfortunately very weak. It can be concluded that the N and C domains are not suitable candidates to be used as epitope-based synthetic peptide vaccines.

A novel strategy that increases the immunogenicity of epitopes was suggested by Chen *et al.,* (1999a). By making use of 'epitope' or 'multi-epitope' vaccines and presenting selected neutralisation-specific epitopes to the immune system, a high titre of epitope-specific antibodies could be elicited. Chen *et al.,* (1999a) found that their ELDKWA and GPGRAFY tetramer epitope vaccines induced an epitope-specific responses that was 2-4 fold higher than the levels induced with synthetic peptide vaccines, consisting of the C

and N domains respectively. Xiao *et al.,* (2000b) reported that the immunogenicity of the ELDKWA epitope is the greatest when presented by the ELDKWA-tetramer-epitope vaccine, C- (ELDKWA)4-BSA. This vaccine was capable of inducing a strong antibody response against ELDKWA ranging from 1:12800 to 1: 25600 in mice. In addition, Xiao *et al.,* (2000c) found that the RILAVERYLKD-dimer epitope vaccine, C(RILAVERYLKD)2-BSA, also induced a strong epitope-specific antibody response in mice (1:25600). In rabbits, the titres of the ELDKWA-specific antibodies elicited by the ELDKWA epitope vaccine were found to be lower (1:6400). However, this antibody response was still significantly higher than the antibody response (1:400) to both ELDKWA and RILAVERYLKD induced by the rgp41 subunit vaccine in mice and rabbits. Besides the high levels of antibody induced by 'epitope' vaccines, Xiao *et al.,* (2000c) also concluded that the antibodies elicited were able to recognise and bind their neutralising epitopes on the N and C domains of gp41. ELDKWA-specific antibodies present in the mouse and rabbit antisera were found to interact very strongly with the P2 peptide sequence (1:25600 dilution), while the RILAVERYLKD specific antibodies in mouse antisera interacted very strongly with Peptide P1 (1: 102400 dilution). P1 and P2 are peptides whose amino acid sequence corresponds to the N and C-domains of gp41 respectively. In contrast, antibodies raised by the rgp41 subunit vaccine bound very weakly to the peptides P1 and P2. These results indicate that the configuration of the epitope presented by the epitope vaccines is very similar to their native conformation, resulting in the induction of neutralising antibodies capable of binding to native epitopes. The failure of the rgp41 subunit vaccine to elicit such strongly binding neutralisation antibodies could be due either to the obscuring of the ELDKWA and RILAVERYLKD epitopes by env glycoslylation and oligomerisation or due to their lack of immunogenicity, as discussed previously.

All the epitope vaccines mentioned thus far have consisted of a single epitope, repeated a number of times. Multi-epitope vaccines, which are composed of a number of different potent neutralising epitopes, show an even greater potential (Ding *et al.,* 2000; Lu *et al.,* 2000a; Lu *et al.,* 2000b). Combinations of human mAbs with different epitope specificities have shown additive or synergistic neutralisation of TCLA or primary HIV-1 isolates *in vitro* (Buchbinder *et al.,* 1992; Laal *et al.,* 1994; Tilley *et al.,* 1992; Vijh-Warrier *et al.,* 1996). A combination of the anti-HIV neutralizing monoclonal antibodies 2F5, 2G12 and HIV Immuno globulin, when passively administered, are able to protect

macaques against vaginal pathogenic SHIV challenge (Mascola *et al.*, 2000). Most notably, infusion of all three these monoclonal antibodies provide a greater degree of protection than any single antibody administered individually. These results indicate that mAbs with different epitope specificities and at appropriate concentrations act synergistically on HIV-1 leading to more potent neutralisation and better protection. Eliciting specific neutralisation antibodies in susceptible individuals by making use of a multi-epitope vaccine would also be more advantageous than using polyclonal antibody preparations for passive immunotherapy (Montefiori *et al.,* 1998). These authors found that a triple combination of human mAbs 2F5, 2G12 and IgG1b12 *in vitro* was able to neutralise primary HIV-1 strains at concentrations lower than a polyclonal antibody preparation. This approach may also eliminate the unpredictable influence of infection-enhancing antibodies or antibodies that interfere with the neutralising antibodies that occur in the polyclonal sera of immunised or infected individuals (Li *et al.,* 1998).

If a high titre of several potent neutralising antibodies could be elicited by a multi-epitope vaccine, this could enable 100% neutralisation of heterologous primary HIV challenge strains. Additional support for the multi-epitope vaccine comes from the prospect that a combination of epitopes would serve as a preventative measure against viral mutation. Should a single epitope mutate to allow viral escape from epitope-specific neutralising antibodies induced by the vaccine, antibodies directed against additional epitopes will still be able to suppress viral replication *in vivo* (Mo *et al.,* 1997).

Ding *et al.,* (2000) were the first authors to design such a multi-epitope vaccine. The CG-GPGRAFY-G-ELDKWA-G-RILAVERYLKD peptide was conjugated to the carrier protein keyhole limpet hemocyanin (KLH) and used to immunise mice. Strong RILAVERYLKD and ELDKWA-specific neutralising antibodies, in the order of 1:51 200 and 1:12 800 respectively, were raised by the vaccine while only a moderate antibody response of 1: 1600 to the GPGRAFY epitope was observed. The epitope-specific antibodies that were raised were also able to bind strongly to their corresponding N domain, C domain and V3 loop peptides as well as to rgp41. This demonstrates the ability of the elicited antibodies to recognise native epitopes on recombinant soluble gp41. Lu *et al.,* (2000a) also constructed two multi-epitope vaccines, PI-BSA and PII-BSA. The PI peptide, composed of C-G-(ELDKWA-GPGRAFY)2-K and the PII peptide (CG-GPGRAFY-G-ELDKWA-G-RILAVERYLKD) were both conjugated to carrier protein

BSA. Following immunisation of rabbits, PI-BSA induced very high levels of ELDKWA-specific antibody (titre 1:102 400) and high levels of GPGRAFY specific antibodies (1:12 800-25 600). PII-BSA induced high levels of antibody specific to ELDKWA and RILAVERYLKD (titre 1: 12 800- 25 600) but a weak response to GPGRAFY. The levels of epitope-specific antibodies induced by these multi-epitope vaccines in rabbits were much greater than 1μg/ml, a level considered to confer long term protection against some viruses (Purtscher *et al.,* 1994). As before, the antibodies elicited were able to recognise their epitopes on corresponding peptides and rgp41. The same authors, Lu *et al.,* (2000b), also tested these multi-epitope vaccines in mice. Similar results were obtained with the PII-BSA vaccine, which induced a strong antibody response against ELDKWA (1:6400) and RILAVERYLKD (1: 102 400) but a weak response of 1:400 to GPGRAFY. The PI-BSA vaccine induced a strong ELDKWA specific antibody response (1:12 800) but a weak response to GPGRAFY. The GPGRAFY epitope seems to have a weak immunogenicity even when presented by the epitope-vaccine strategy. Lu *et al.,* (2000a) suggested that a epitope vaccine with repeated epitopes could elicit higher titres of these epitope-specific antibodies than a vaccine containing only one copy of the epitope. This certainly seems true for the epitope GPGRAFY following the research done by Yu *et al.,* (2000) that suggests that the C- (GPGRAFY)4 multi-epitope vaccine is able to induce high levels of GPGRAFY specific antibodies in rabbits and mice.

1.5.5 AN HIV-1 VACCINE FOR SUB-SAHARAN AFRICA

As HIV-1 subtype B is the predominating subtype in North America and Europe, much of the research concerning an HIV-1 vaccine has centred on this clade. However, HIV-1 subtype B is only responsible for 16% of global infections, while HIV-1 subtype C is currently the most frequently transmitted subtype and accounts for 54% of people infected world-wide (UNAIDS & WHO, Geneva, 1998). The research priorities, particularly in developing countries, have therefore shifted to the design of a vaccine based on subtype C. Various candidate viral vaccines against HIV-1 subtype C are currently in the process of being developed. HIV-1 subtype C genes have been inserted into NYVAC (an attenuated vaccinia virus), MVA, Venezuelan equine encephalitis virus (VEE), adeno-associated virus as well as the Semliki Forest and sindbis viruses (reviewed in Johnston & Flores, 2001).

The current perception is that subtype-specific HIV-1 vaccines are the only way to counteract the subtype specific HIV-1 epidemics around the world (Morris *et al.*, 2000). Thus, the design of an HIV-1 subtype C vaccine should incorporate, amongst other things, strong neutralising subtype C epitopes. As much work has been carried out with the subtype B ELDKWA and RILAVERYLKD neutralising epitopes, their correlative amino acid sequences in subtype C isolates may also display this protective capability (Huismans, personal communication). Aligned amino acid sequence data of various subtype C viral isolates has shown the equivalent consensus sequences to be ALDSWQ and RVLAIERYLKD (unpublished data). As these subtype C amino acid sequences are not identical to those of the subtype B epitopes, the immunogenicity of these and the epitope-equivalent sequences of the subtype C strain Du 151, ALDSWK and RVLAIVERYLKD (van Harmelen *et al.*, 2001), is yet to be established. The HIV-1 subtype C strain Du 151 is a representative strain of the current HIV-1 viral population in South Africa (Williamson *et al.*, 2000) and has been approved for research pupouses. If its epitope-equivalent sequences ALDSWK AND RVLAIVERYLKD are able to elicit a protective neutralising immune response in mice they will subsequently be incorporated into the design of an HIV-1 vaccine for Southern Africa.

1.6 AIMS OF THIS STUDY

The research objectives in this study will focus on two different issues that will be addressed in parallel. The first objective will focus on the use of a novel particulate protein vaccine system for the presentation of possible neutralising epitopes to the immune system and the second on the development of a live poxviral vector system for the delivery of neutralising and other immunologically important epitopes into target cells for subsequent expression and immunological presentation.

The long term aim remains to explore the AHSV-9 VP7 epitope presentation system and the LSDV live viral vector delivery system as two different vaccine strategies, particularly in view of the development of an HIV-1 vaccine. In this dissertation the focus was on the following shorter aims:

1. To express the HIV-1 subtype C Du151 strain gp41 epitopes ALDSWK and RVLAIERYLKD on the surface of AHSV-9 VP7 crystalline particles- the longer-

term aim being to synthesise larger quantities of these particulate structures that can be utilised to assess the immune response against these peptides

2. To further evaluate the efficiency of the LSDV bi-directional promoter in expressing chimeric VP7 proteins by utilising the late element of the bi-directional promoter to determine expression levels.

CHAPTER 2

CONSTRUCTION OF A RECOMBINANT BACULOVIRUS AND EXPRESSION OF THE CHIMERIC AHSV-9 VP7 PROTEIN

2.1 INTRODUCTION

As reviewed in Chapter 1, the HIV-1 subtype B amino acid sequences ELDKWA and RILAVERYLKD are recognised epitopes capable of inducing a humoral immune response. In contrast, the immunogenic status of their correlative subtype C sequences is yet to be established. Although the immunogenicity of the subtype C epitope-equivalent sequences is unknown, for want of a more manageable term, they will forthwith be referred to as epitopes.

The first step in determining the immunogenicity of the HIV-1 C strain DU 151 epitope sequences, ALDSWK and RVLAIERYLKD, was to elucidate a strategy of presenting them to the immune system. It is well known that small peptides need to be conjugated to larger carrier proteins, such as BSA, in order to elicit a humoral immune response. Alternatively, insertion of the peptides into various protein molecules has also been employed for presentation to the immune system (discussed in Chapter 1).

A novel approach, which may possibly result in the induction of a strong epitope-specific neutralising immune response, will be taken in the current study. AHSV-9 VP7 crystalline particles shall be utilised as a particulate protein presentation system. This strategy has numerous advantages. Firstly, the AHSV-9 VP7 protein has been found to tolerate an insertion of approximately 100 amino acids into its top domain without the loss of its trimeric structure and ability to form crystals (Maree, 2000). Peptides less than 100 amino acids in length could thus easily be presented to the immune system on a scaffold of VP7 crystals. The insertion site chosen for this study is a RGD motif (aa 175-180) present on the top domain of VP7 (reviewed in Chapter 1). It is one of five hydrophilic regions on the surface of VP7 (Huismans, personal communication). Amino acids into this site would subsequently loop out of the VP7 protein and be well exposed to the

immune system. Consequently peptides containing the ALDSWK and RVLAIERYLKD epitopes as well as their flanking regions (54 and 57 amino acids in size respectively) will be inserted into position 177 of the AHSV-9 VP7 protein. Another advantage to utilising this system is that two epitopes may be inserted in combination and be presented to the immune system on a single VP7 protein. This approach could possibly result in a higher titre of neutralising antibodies being induced than by either of the epitopes individually. Consequently a combination of the subtype C epitopes ALDSWK and RVLAIERYLKD as well as their flanking amino acids (a total of 105 amino acids) will also be inserted into position 177. Although clearly above the 100 amino acid insertion limit, it will be interesting to determine whether the VP7 trimeric structure is able to tolerate such a large insertion without the loss of ability to form insoluble crystals.

Unique in this strategy is the presentation of epitopes in combination with their flanking amino acids to the immune system. In previous studies only short amino acid sequences comprising of the specific epitopes have been presented to the immune system via various particulate protein strategies. Here it was hypothesised that should epitopes be presented in combination with their flanking amino acids they may be able to attain a more immunologically correct configuration and subsequently induce higher titres of neutralising antibodies (Huismans, personal communication). Although the presentation of epitopes in larger protein conformations on the surface of a AHSV-9 VP7 protein crystalline scaffold may not necessarily prove to be more successful at inducing high titres of antibodies than any of the other protein particulate presentation strategies, this approach must nevertheless be explored.

In order to pursue this strategy, large quantities of the chimeric VP7 proteins, mentioned above, must be synthesised for immunological studies in order to determine whether the inserted peptides are immunogenic. These large quantities of the chimeric VP7 proteins can be obtained by making use of recombinant viruses. During their replicative cycle, these viruses express the inserted foreign genes in their relevant host cells after which foreign proteins can be harvested. The advantage of utilising recombinant eukaryotic viruses, such as poxviruses and baculoviruses is that the foreign protein expressed in eukaryotic host cells is correctly modified after translation and mimics the biological action of the naturally occurring protein.

The construction of recombinant poxviruses allowing initially the expression of the chimeric VP7 gene for use in immunological studies and their eventual use as live poxviral systems for the delivery of these chimeric proteins, would be ideal. However, the generation of recombinant poxviral vectors via homologous recombination is a long process, involving repeated rounds of purification of recombinant poxvirus from contaminating wild type virus. It would thus not be advantageous to undertake the time-consuming process of generating recombinant poxviruses, until it is determined whether the ALDSWK and RVLAIERYLKD sequences are indeed immunogenic epitopes.

An alternative to the generation of recombinant poxviruses, is the construction of recombinant baculoviruses. The Bac-to-Bac™ system, described by Luckow *et al.*, (1993) improves the ease with which baculovirus recombinants are generated. Recombinant baculovirus generation via the long process of homologous recombination between the recombinant transfer vector and a parent *Autographa californica* nuclear polyhedrosis viral (AcNPV) genome is circumvented. Instead this process is replaced by a system enabling site-specific transposition of a DNA cassette (containing the foreign gene) into a baculovirus shuttle vector (bacmid), which is propagated in *E.coli* (Fig 2.1).

The bacmid is a recombinant *Autographa californica* nuclear polyhedrosis viral (AcNPV) genome. It contains a mini-F replicon, a kanamycin resistance marker and a *lac* Zα gene. Inserted into the N-terminus of the Lac Z gene is the attachment site for the bacterial transposon Tn7 (mini-*att* Tn7), which does not disrupt the reading frame of the Lac Zα peptide. The bacmid is thus able to replicate in *E.coli* DH10 Bac cells as a plasmid that confers resistance to kanamycin and also complements a *lacZ* deletion on the bacterial chromosome to form colonies that are blue (Lac^+) in the presence of the chromogenic substrate X-gal and inducer IPTG.

The mini-Tn7 in the pFASTBAC donor plasmid contains an expression cassette consisting of a Gen^r gene, a baculovirus-specific promoter (AcNPV polyhedrin promoter), a multiple cloning site (MCS) and a SV40 poly A sequence inserted between the left and right arms of Tn7. Genes to be expressed are inserted into the MCS of the plasmid downstream from the polyhedrin promoter. Recombinant bacmids are generated by transposing the mini-Tn7 element from the recombinant pFASTBAC donor plasmid to the mini-attTn7 attachment site on the bacmid. This is only possible if the Tn7

transposition functions are provided *in trans* by a helper plasmid (pMON7124). When the mini-Tn7 is inserted into the mini-attTn7 attachment site on the bacmid, this disrupts expression of the lacZα peptide. The bacterial colonies containing the recombinant bacmid will subsequently be white while those that contain parental bacmid DNA will be blue. *E.coli* colonies containing the recombinant bacmid DNA are thus identified by antibiotic and blue/white selection. The purely recombinant bacmid DNA is isolated and transfected into insect cells giving rise to recombinant baculoviruses expressing the gene of interest.

The Bac-to-Bac™ system thus allows for the rapid and efficient generation of recombinant baculoviruses. In addition to increasing the frequency of recombination, this system also eliminates the need for repeated rounds of plaque purification in order to purify recombinant virus from residual wild type (wt) virus. As large quantities of chimeric VP2 protein can be harvested from cultured insect cells infected with recombinant baculovirus (Roy *et al.*, 1996) and the system is well established at the UP genetics Department, recombinant baculoviruses were constructed.

To construct the recombinant baculoviruses expressing the chimeric VP7 proteins recombinant pFASTBAC vectors, containing chimeric AHSV-9 VP7 sequences, will have to be transposed into DH10 Bac *E.coli* cells and the recombinant bacmid DNA isolated. The bacmid DNA subsequently transfected into Sf9 insect cells will, presumably, give rise to recombinant baculoviruses. Expression of the chimeric VP7 proteins will be determined by polyacrylamide gel electrophoresis.

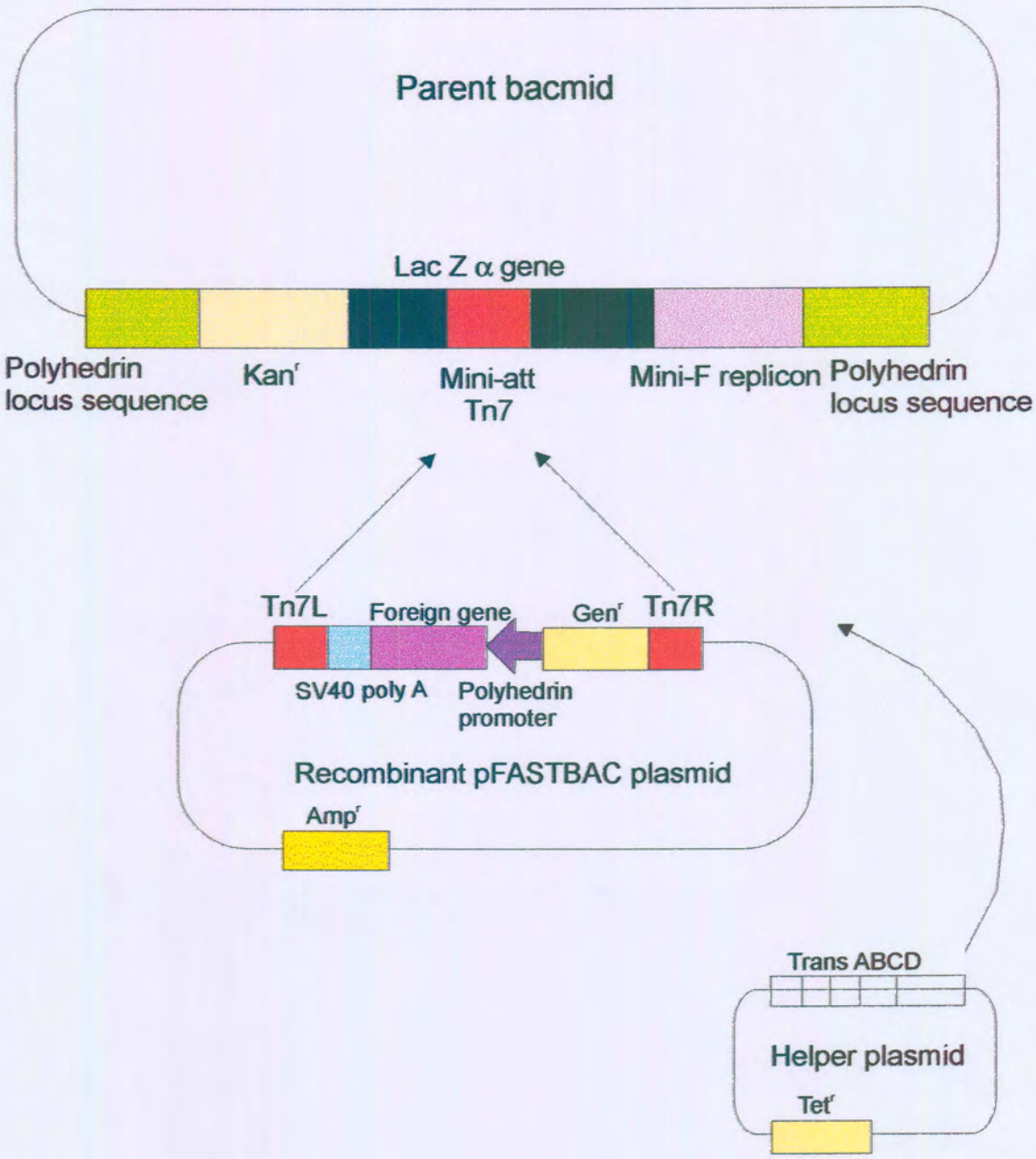

Fig 2.1 Schematic representation of the site-specific transposon-mediated insertion of foreign genes into the baculovirus genome propagated in *E.coli*, resulting in the generation of recombinant baculovirus shuttle vectors (bacmids). Donor plasmid containing the foreign gene is used to transform *E.coli* cells containing the helper plasmid and bacmid. The mini-Tn7 on the donor plasmid can transpose to the parent bacmid in *E.coli* when transposition functions are provided *in trans* by the helper plasmid.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of competent *Escherichia coli* cells

The calcium chloride method of preparing competent bacterial cells, originally described by Mandel & Higa (1970), was used. This method is based on the principle that bacterial cells are made more susceptible to the uptake of foreign DNA (competent) when exposed to calcium ions at a low temperature. A 2 ml overnight culture of *E. coli* XL-1 Blue cells was used to inoculate 100 ml of sterile Luria-Bertani (LB) broth (1% bactotryptone (m/v), 0.5% bactoyeast (m/v), 1% NaCl (m/v)), prewarmed to 37°C. The culture was grown to logarithmic (log) phase ($O.D_{550}$ = 0.45) by incubation with shaking at 37°C. After approximately 4 hr, the culture was placed on ice for 10 min to inhibit mitosis (as described by Sambrook *et al.*, 1989). Cells were collected by centrifugation at 2000 rpm for 10 min at 4°C and carefully resuspended in half the original volume of ice-cold, freshly prepared 50 mM $CaCl_2$. After 30 min on ice, the cells were again pelleted by centrifugation as described above and resuspended in 1 ml of 50 mM $CaCl_2$. The cells to be used directly for transformation were incubated on ice for 1 hr before the event. The remainder of the cells were aliquoted into 300 µl fractions and sterile glycerol was added to a final concentration of 15%. These cells were frozen away at - 70°C.

2.2.2 Transformation

Approximately 250 ng plasmid DNA was added to 100 µl of XL-1 Blue competent cells on ice. As a positive control, a known quantity of supercoiled plasmid was also added to 100 µl of competent cells in order to determine the transformation efficiency or competency of the *E.coli* cells. The tubes were lightly tapped and left on ice for 30 min to allow for DNA adsorption. The cells were then subjected to heat-shock at 42°C for 90 sec and cooled on ice for 2 min. Nine-hundred µl of sterile Luria-Bertani (LB) broth, prewarmed to 37°C, was added to each of the mixtures and the cells were incubated at 37°C for 1 hr with shaking. One tenth of each mixture was plated onto 1.2% (m/v) LB broth agar plates containing 100 µg/ml ampicillin (amp) and 12.5 µg/ml tetracycline (tet), using a spreading technique as described Sambrook *et al.*, (1989). The plates were incubated at 37°C overnight. Tetracycline and ampicillin resistant colonies were selected for screening.

2.2.3 Plasmid DNA isolation

Plasmid DNA extraction from liquid cultures of bacterial cells was done according to the alkaline lysis method of Birnboim & Doly (1979), as described in Sambrook *et al.*, (1989). Several

bacterial colonies on the LB plates containing ampicillin and tetracycline were individually picked with sterile toothpicks and inoculated into 3 ml of sterile LB broth containing 100 μg/ml ampicillin and 12.5 μg/ml tetracycline. The cultures were incubated overnight at 37°C with shaking. The inoculated colonies were also restreaked onto identical agar plates (replica plated) and left at 37°C overnight.

During the small scale plasmid purification procedure, bacterial cells were harvested by centrifugation at 5000 rpm for 10 min. Following the removal of the supernatant, the cells were resuspended in 100 μl of ice-cold Solution I (25 mM Tris-HCl, pH 8.0, 50 mM glucose, 10 mM EDTA), placing the cells in an isotonic environment. After 5 min on ice, 200 μl of freshly prepared Solution II (0.2 NaOH, 1% SDS) was added, the tubes gently inverted 5 times and incubated on ice for 2 min. This step allows for the controlled and gentle lysis of the bacterial cells as well as the denaturation of proteins, chromosomal and plasmid DNA. One hundred and fifty μl of 3 M NaOAc pH 4.8 was added to the reaction and vortexed vigorously, resulting in the reannealing of the plasmid DNA strands as well as the precipitation of chromosomal DNA, high molecular mass RNA and SDS-denatured proteins. After 5 min on ice, the chromosomal DNA and insoluble proteins were pelleted by centrifugation at 13000 rpm for 10 min and the supernatant transferred to a new tube. The plasmid DNA was precipitated by the addition of a tenth of the volume of 3 M NaOAc pH 4.8 and 2.5 volumes of 96% ethanol. The reactions were incubated at -20°C for 1 hr. The plasmid DNA was recovered by centrifugation at 13000 rpm for 20 min, the DNA pellet washed with 70 % ethanol and centrifuged at 13000 rpm for 5 min. The plasmid DNA was dried in a Speedvac vacuum centrifuge (Savant) and resuspended in 30 μl dd$H_2$0. In order to remove contaminating residual proteins, a phenol-chloroform extraction was performed. The volume of the plasmid solution was scaled up to 400 μl, followed by the addition of an equal volume of 1:1 phenol-chloroform. The sample was vortexed for 10 sec and centrifuged at 13000 rpm for 15 min. The supernatant was transferred to a clean eppendorf tube and half of its volume of chloroform was added. This step is included to remove all residual phenol contamination. After vortexing and centrifugation at 13000 rpm for 5 min, the supernatant was transferred to a new tube for ethanol precipitation of the plasmid DNA, as described above. The plasmid DNA of several candidate clones was isolated using this procedure, analysed by 1% agarose gel electrophoresis and stored at -20°C.

Characterisation of plasmid DNA was performed by restriction endonuclease digestion described in section 2.2.7. After identification of the desired plasmid, a large-scale plasmid extraction procedure was undertaken using the Nucleobond AX kit (Macherey-Nagel). The kit is based on a slightly modified alkaline lysis method.

2.2.4 DNA sequencing

Prior to the assembly of all sequencing reactions, the double stranded template DNA was purified using the High Pure PCR product purification kit (Roche) and its concentration determined by making use of the following equation:

$$\text{Concentration} = A_{260nm} \times \text{sample dilution} \times 50\mu g/ml$$

The purity of the template DNA was also assessed by employing the ration A_{260nm}/A_{280nm}. Sequencing reactions were performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit version 2.0 (Applied Biosystems). Sequencing was accomplished by using the oligonucleotide primers described in Table 2.1, Full volume sequencing reactions were prepared in 0.2 ml tubes (Simport) and contained 250 ng plasmid DNA, 3.2 pmol of a specific primer and 4 µl Terminator Ready Reaction mix (supplied with the kit) in a final volume of 10 µl. Reactions were cycle sequenced in the Perkin Elmer GeneAmp PCR system 9600. The reaction conditions: 96°C for 10 sec, 50°C for 10 sec and 60°C for 4 min were repeated for 25 cycles. After cycle sequencing, the extension products were purified with the Ethanol/NaOAc precipitation protocol in microcentrifuge tubes, as detailed in the manufacturer's manual. This step is included to remove unincorporated dye terminators so that the early part of the sequences is not obscured when samples are analysed by electrophoresis. The entire contents of each reaction were pipetted into 1.5 ml microcentrifuge tube and the following reagents added: 10 µl deionized H_20, 2 µl 3 M sodium acetate pH 4.6 and 50 µl 95% ethanol. After a 10 min incubation period on ice to precipitate the extension products, the reactions were centrifuged at 12000 rpm for 20 min. The supernatant was aspirated and discarded to ensure the complete removal of unincorporated dye terminators. The pellet was washed with 250 µl 70% ethanol and centrifuged under the same conditions. The supernatant was removed and the pellet dried in a vacuum centrifuge for 10-15 min. Each sample pellet was resuspended in 6 µl loading buffer (deionised formamide, 25 mM EDTA pH 8.0 with blue dextran (50mg/ml)), denatured at 95°C for 2 min and placed on ice prior to loading into the sequencing gel. Samples were loaded and extension products separated by electrophoresis in the ABI Prism 377 DNA sequencer, according to the manufacturer's instructions.

Table 2.1 Primers used to sequence transfer vector, pFASTBAC-VP7-mutant 177

Primer	Sequence	Target
Forward primer pol HF	5'd(TTCCGGATTATTCATAC C)	pFASTBAC polyhedron promoter (3997-4014 bp)
Reverse primer SV 40	5'd(CAGGTCGACTTCGGATC TCCTAGGCTC)	pFASTBAC SV40 poly A & T region (4413-4430 bp)

2.2.5 Design of primers

In order to amplify the sequences encoding the epitopes RVLAIERYLKD and ALDSWK from the HIV-1 subtype C strain Du151 gp160 gene, two pairs of PCR primers were specifically designed. The primers, designed using the gp 160 DNA sequence data obtained from the NIV, are described in Table 2.2 and depicted in Fig 2.2.

Table 2.2 Primers utilised in PCR to obtain desired inserts

Primer	Sequence	Tm $4(G+C)_n + 2(A+T)_n$	Target
RILA-FW	5'd(CCCAAGCTTGC TATAGAGGCGCAAC AG)	82°C	HIV-1subtype C strain Du151 gp160 (1615-1632 bp)
RILA-RV	5'd(CCCGTCGACGA ATTCCACAGCAGTG GTGCAGAT)	104°C	HIV-1subtype C strain Du151 gp160 (1750-1767 bp)
ALD-FW	5'd(CCCAAGCTTGA ATTCAGGTTACTTG AAGACTCG)	96°C	HIV-1subtype C strain Du151 gp160 (1873-1890 bp)
ALD-RV	5'd(CCCGTCGACGC CTCCTACTATCATG AT)	84°C	HIV-1subtype C strain Du151 gp160 (1999-2016 bp)

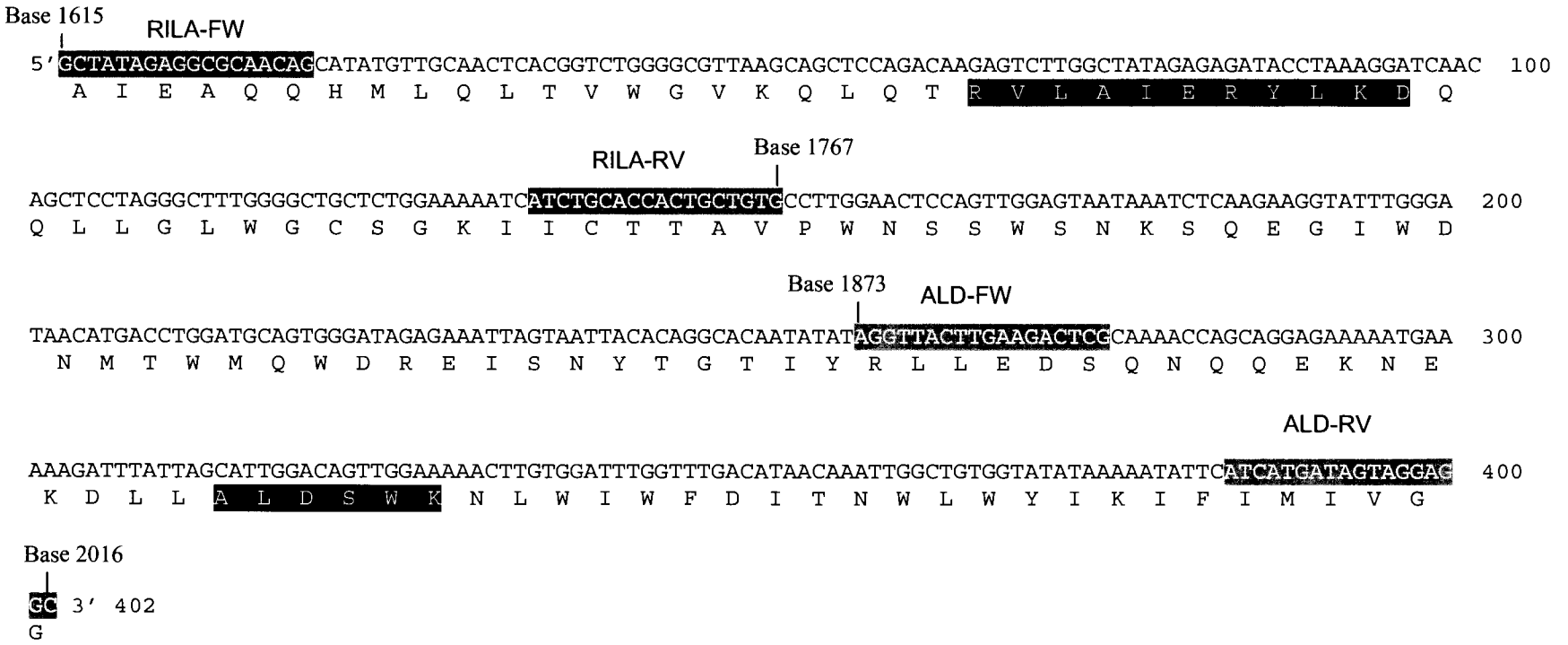

Fig 2.2 A partial nucleotide sequence (1615 bp-2016 bp) of the HIV-1 subtype C strain Du 151 gp 160 gene and the encoded amino acid sequence. Depicted are the primer pairs designed to amplify sequences encoding the epitope-equivalent regions RVLAIERYLKD and ALDSWK. The nucleotides highlighted in grey are the sequences of the primers used to amplify the RVLAIERYLKD and ALDSWK-encoding sequences (highlighted in black) and their corresponding adjacent amino acids sequences. The sequence amplified by the RILA primer pair is 152 bp in size while that amplified by the ALD pair is 143 bp.

2.2.6 Polymerase chain reaction

The primers, described in Table 2.2, were used in specific pairs to amplify certain segments from the template plasmid DNA, as depicted in Table 2.3. The 50 µl PCR reactions contained 250 ng plasmid DNA, 100 pmol of each specific primer, 50 µmol of each dNTP, 1.5 mM $MgCl_2$ (Promega), 5 µl of 10 × Mg-free thermophilic DNA Polymerase buffer (Promega) and 2.5 U of Taq DNA Polymerase enzyme (Promega). A negative control reaction, into which no template DNA had been added, was also prepared to test for possible contamination during the preparation of the reactions. The reactions were performed in a Perkin Elmer GeneAmp PCR system 9600 machine. The reaction conditions were as follows: plasmid DNA was denatured at 95°C for 2 min, followed by 30 cycles of denaturation (95°C for 1 min), primer annealing (72°C for 1 min) and elongation (72°C for 2 min). A final elongation step of 4 min at 72°C was also performed. The PCR products were analysed electrophoretically on a 1% agarose gel with the DNA molecular weight markers DNA Molecular Weight marker II (λII) (Roche) and PhiX174/*Hae* III (ϕX) (Promega). PCR products were purified using the High Pure PCR product purification kit (Roche).

Table 2.3 Sets of primers used, regions amplified and sizes of the expected amplification products

Primer set	Region amplified	PCR product size (bp)
RILA-FW + RILA-RV	HIV-1subtype C strain Du151 gp160 (1615-1767 bp)	176
ALD-FW + ALD-RV	HIV-1subtype C strain Du151 gp160 (1873-2016 bp)	167

2.2.7 Restriction endonuclease digestion

For cloning purposes, both the PCR products and transfer vector were digested with specific restriction endonucleases (Roche). The double digestion reactions were performed in a final volume of 50 µl, containing either 5 µg purified PCR product or 4 µg vector DNA, and 10 Units (U) of each enzyme. Each digestion was performed for 3 hr at the optimum temperature for each enzyme and containing specific salt buffers, as recommended by the manufacturer. The digested DNA products were purified by means of the High Pure PCR purification kit (Roche) and analysed by 1% agarose gel electrophoresis.

2.2.8 Ligation of vector and insert

Sticky-end ligation of the insert DNA to the linearised vector was accomplished by using 1U of T7 DNA ligase (Boehringer Mannheim) in a 20 µl reaction with a final 1 × concentration of ligation buffer (660 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM DTT, 10 mM ATP, pH 7.5). T4 DNA ligase catalyses the formation of phosphodiester bonds between adjacent 3' hydroxyl and 5' phosphate groups on the ends of DNA molecules. The insert: vector molar ratio was the recommended 3:1 ratio. The reactions were incubated overnight at 16°C. A control ligation reaction containing only linearised vector DNA was prepared to determine whether restriction enzyme digestion of the vector had been complete.

2.2.9 Transformation

Half of the volume of the ligation reactions were each added to 100 µl competent XL-1 blue cells on ice. The transformation protocol followed was as described in section 2.2.2. Tetracycline and ampicillin resistant colonies were selected for screening.

2.2.10 Colony blot

A rapid procedure for the identification of recombinant plasmid is *in situ* hybridisation to bacterial colonies (Grunstein & Hogness, 1975). The following procedure was followed as described by Sambrook *et al.*, (1989). A dry Hybond N^+ nylon membrane (Amersham) was placed on a plate containing bacterial colonies and carefully peeled off. In this way the bacterial colonies were lifted onto the membrane. The agar plates were re-incubated at 37°C for a few hours to allow regrowth of the colonies for future picking of positive clones. The membrane was then placed, colony side up, on four pieces of Whatmann 3 MMchr filter paper saturated with 10% SDS for 3 min. This treatment limits the diffusion of plasmid DNA during denaturation and neutralisation steps and results in a sharper hybridisation signal. The membrane was lifted and placed on a second stack of filter paper, saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl), for 5 min. The membrane was transferred to a filter paper stack saturated with neutralisation solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.4) for 5 min. After the membrane was placed on filter paper saturated with 2 × SSC (30 mM Na_3citrate.$2H_2O$, 150 mM NaCl pH 7.0) for 5 min, it was placed, colony side up, on dry filter paper for 30 min at room temperature to dry. It is important to note that the filter paper should be saturated with the appropriate solutions, however, too much fluid will cause the bacterial colonies to swell and diffuse during lysis making it difficult to identify recombinant clones. Also, when transferring the membrane, no solutions should make contact with the side

containing the colonies. Once dry, both sides of the membrane were exposed to UV illumination for 3 min, thereby fixing the bacterial DNA onto the membrane.

Non-radioactive labelling of DNA probes

The PCR products, resulting from section 2.2.6, were labelled using the DIG DNA labelling and Detection kit (Boehringer Mannheim) and were utilised as probes. This non-radioactive system uses digoxigenin (DIG), a steroid hapten, to label DNA probes for hybridisation to target DNA and subsequent colour detection by enzyme immunoassay. DIG-labelled DNA probes are generated enzymatically using the random-primed labelling method. The method is based on the hybridisation of random oligonucleotides to denatured DNA template. The Klenow enzyme uses the 3' hydroxyl termini of the hybridised oligonucleotides as primers and a mixture of deoxyribonucleotides, including DIG-11-dUTP, to synthesise the complementary DNA strand. As a result of elongation, DIG UTP is incorporated every 20-25 nucleotides into the newly synthesised DNA.

For DNA labelling, 0.32-1 μg DNA template was diluted to a final volume of 15 μl and denatured at 95°C for 10 min. The reactions were quickly chilled by placing them on ice. To each reaction, now containing single-stranded DNA, the following reagents were added: 2 μl of 10 × Hexanucleotide mix, 2 μl of 10 × dNTP labelling Mix (1 mM dATP, 1 mM dGTP, 1 mM dCTP, 0.65 mM dTTP, 0.35 mM DIG-11-dUTP pH 7.5) and 1 μl of Klenow enzyme (labelling grade, 2 U/μl). The reactions were incubated at 37°C overnight after which they were placed on ice and terminated by the addition of 2 μl of a 0.2 M EDTA pH 8.0 solution. To purify the probes, 2.5 μl 4 M LiCl and 75 μl 96% ethanol were added to each reaction to precipitate the labelled probe. The reactions were incubated at -70°C for 30 min. Labelled DNA was collected by centrifugation at 10000 rpm for 30 min, after which the pellet was washed with 100 μl 70% ethanol and recovered with centrifugation under the same conditions. The pellet was briefly dried in a vacuum centrifuge and resuspended in 50 μl ddH_2O. Probes can be stored at -20°C for up to 1 year. Labelled probes were quantified using the direct detection method, as described in the DIG instruction manual. The yield of DIG-labelled DNA must be determined if an optimal and reproducible hybridisation result is to be obtained. If the probe concentration is too high in the hybridisation reaction, this results in background while too low a probe concentration leads to weak hybridisation signals. The DIG-labelled PCR probes were serially diluted and dot blotted onto a small piece of nylon membrane. Defined dilutions of DIG-labelled control DNA, supplied by the kit, were also blotted onto the membrane as standards. The nylon membrane was subjected to an immunological detection procedure with the anti-digoxigenin AP antibody conjugate and freshly prepared colour substrate solution, as described in the manufacturers manual. By comparing the resulting colour

intensities of the probe dilution series with that of the control DNA the concentrations of the DIG-labelled PCR products was calculated by taking the dilution factors into account.

Hybridisation of probes with transferred DNA

The Hybond N^+ nylon membranes (Amersham), onto which the plasmid DNA had been transferred and fixed, were soaked in 5 × SSC (75 mM Na_3citrate.$2H_2O$, 375 mM NaCl pH 7.0) for 2 min. The membranes were transferred into 20 ml hybridisation solution (5 × SSC, 0.1% (w/v) N-lauroylsarcosine, 0.2% (w/v) SDS, 1% Blocking reagent (supplied in DIG kit)) and prehybridized for 2 hr at 65°C. The DIG-labelled probes were denatured at 95°C for 10 min and rapidly cooled on ice. The pre-hybridisation solution was replaced by 1.75 ml of fresh hybridisation buffer, which had been pre-heated to 65°C. The appropriate denatured probes were added to each hybridisation solution, ensuring a final quantity of 50 ng of each probe. A small piece of nylon membrane, onto which DNA containing sequences homologous to the probe had been spotted, served as a positive control and was included in the hybridisation reactions. Hybridisation was allowed to proceed overnight at 65°C. Membranes were removed from the sealed plastic bags and were subjected to post-hybridisation washes with various stringencies. Low stringency washes were performed twice, for 5 min each, at room temperature in 2 × SSC, 0.1% SDS and with constant agitation (as recommended by the manufacturer). The washes of a higher stringency were performed twice, for 15 min each, at 42°C with 0.1 × SSC, 0.1% SDS under constant agitation.

Immunological detection of hybridised probes

Following the post-hybridisation washes, the membranes were equilibrated for 5 min in Washing buffer (0.3% (v/v) Tween^R 20 in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl pH 7.5)). Membranes were subsequently incubated in freshly prepared 1 × blocking solution (1 × working solution is prepared by diluting the 10 × stock solution 1:10 in maleic acid buffer. The 10 × stock solution is composed of 10% (w/v) blocking reagent, supplied in the kit, in maleic acid buffer) at room temperature with agitation. The anti-digoxigenin Alkaline Phosphatase enzyme conjugate was diluted 1: 5000 in 1 × blocking solution, resulting in a final concentration of 150 mU/ml. The blocking solution was replaced by this antibody solution and the membranes incubated for 30 min, at room temperature, with constant agitation. The membranes were washed twice in Washing buffer, 15 min each with constant agitation, and equilibrated for 2 min in Detection buffer (0.1 M Tris-HCl, 0.1 M NaCl pH 9.5). The colour substrate solution, to be used during the detection procedure, was prepared just prior to its incubation with the membranes by adding 400 µl NBT/BCIP (supplied with the kit) to 20 ml detection buffer. Each membrane was sealed in a

plastic bag, to which 10 ml of the colour substrate solution was added. Membranes were incubated in the dark at room temperature with no agitation, until the desired band colour intensities were achieved. The membranes were washed with dH_2O to stop the colour development reaction and air-dried.

2.2.11 Confirmation of recombinant clones

To confirm the results of the colony blot experiment, plasmid DNA from randomly selected bacterial colonies was used as template DNA in PCR reactions. A pair of primers specific for VP7 (Table 2.4) were utilised and were expected to yield amplification products of a specific size if the template plasmid was recombinant. The PCR reactions contained approximately 100 ng DNA and identical quantities of all the PCR reagents listed in section 2.2.6. The appropriate positive and negative controls were prepared and all reactions were performed in a Perkin Elmer GeneAmp PCR system 9600 machine. The reaction conditions were as follows: DNA template was denatured at 95°C for 2 min. This was followed by 30 cycles of denaturation (95°C for 1 min), primer annealing (55°C for 1 min) and elongation (72°C for 2 min). A final elongation step of 4 min at 72°C was also performed. The PCR products were analysed electrophoretically on a 1% agarose gel with the DNA molecular weight markers.

Table 2.4 Primers used in PCR to identify recombinant clones

Primer	Sequence	Tm $4(G+C)_n + 2(A+T)_n$	Target
Forward primer VP7 FWD1	5'd(TTACGTACCGCA AGGTCG)	56°C	Native AHSV-9 VP7 (440-457 bp)
Reverse primer VP7 RVH1	5'd(GAACCGTGTCT AGCGATC)	56°C	Native AHSV-9 VP7 (781-798 bp)

2.2.12 Sequencing of recombinant clones

One randomly chosen clone of each recombinant was partially sequenced using an oligonucleotide primer described in 2.2.11. The sequencing protocol followed, including the precipitation of the sequencing products and their separation by electrophoresis, was described in section 2.2.4.

2.2.13 Transformation into *E.coli* and transposition

Competent *E.coli* DH10Bac cells were prepared using the DMSO method, as described by Chung & Miller., 1988. Approximately 100 ng of plasmid DNA was added to 100 µl competent cells on ice. Tubes were lightly tapped and incubated on ice for 30 min to allow for DNA adsorption. Cells were heat-shocked for 45 sec at 42°C and placed on ice for 2 min. Nine hundred µl of sterile TSBG medium (LB broth containing 10% (w/v) polyethyleneglycol (PEG), 5% (v/v) dimethyl sulphoxide (DMSO), 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to each tube and the cells incubated at 37°C with medium agitation for 4 hr. The transposition mixtures were each diluted, using the TSBG medium, to 10^{-1} dilutions. One tenth of the mixtures, and their 10^{-1} dilutions, were plated out onto 1.2% m/v LB broth agar plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline and 40 µg/ml IPTG (Isopropyl-β-D-thiogalactopyranoside (Boehringer Mannheim)). One hundred µl of a 2% X-gal (5-Bromo-4-chloro-3-Indolyl-β-D-galactopyranoside (Biosolve)) solution was added to each standard size agarose plate just prior to the plating out of cells. Plates were incubated for at least 24 hr at 37°C to facilitate differentiation between blue and white colonies. Candidate recombinant white colonies were replica-plated onto identical plates and incubated overnight at 37°C to confirm their phenotypes.

2.2.14 Isolation of recombinant bacmid DNA

The protocol for isolating recombinant bacmid DNA is an adaptation of the alkaline lysis method (2.2.3) and is described in the BAC-TO-BAC™ Baculovirus Expression System manual. Several white colonies, their phenotype verified, were individually picked and inoculated into 2 ml sterile LB broth supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin and 10 µg/ml tetracycline. The cultures were incubated at 37°C overnight with shaking and the bacterial cells pelleted by centrifugation at 5000 rpm for 10 min. The supernatant was aspirated and the cell pellet gently resuspended in 300 µl of Solution I (25 mM Tris-HCl, pH 8.0, 50 mM glucose, 10 mM EDTA) at 4°C. Following the addition of an equal volume of Solution II (0.2 M NaOH, 1% SDS), the reactions were gently mixed and incubated at room temperature for 5 min. Three hundred µl of a 3 M KOAc pH 5.5 solution was slowly added, mixing gently. The samples were placed on ice for 10 min and subsequently centrifuged at 13000 rpm for 15 min. The clear supernatant was transferred to a fresh tube containing 800 µl absolute isopropanol at room temperature. Tubes were inverted a few times to mix the contents and the bacmid DNA precipitated by placing the tubes on ice for 10 min. At this stage the samples can be stored at -20°C for up to one month. Following centrifugation at 13000 rpm for 20 min, the DNA pellet was washed with 1 ml 70% ethanol by inverting the tube several times. Samples were centrifuged at 13000 rpm for 10 min after which tubes were transferred to a sterile laminar flow hood and the supernatants aspirated.

The pellet was air-dried briefly for 10 min in a sterile environment and 40 µl sterile dH_2O added to each tube. The DNA was allowed to dissolve for 10 min at room temperature, with occasional tapping at the bottom of the tube. Bacmid DNA was analysed in a 0.5% agarose gel by electrophoresis at a constant voltage of 23V for 12 hr.

2.2.15 Insect cell culture

Spodoptera frugiperda (Sf9) cells were obtained from the NERC Institute of Virology, Mansfield Road, Oxford, UK. The cell culture medium consisted of Grace's Insect Medium, supplemented with 10% Foetal calf serum (FCS), 1.2 × antibiotic-antibiotic solution, all supplied by Highveld Biological, and 0.08% Pluronic F-68 (Sigma). Sf 9 cells were grown as suspension cultures in spinner flasks (Pyrex) at 27ºC with rotational shaking at 114 rpm in a labcon rotational shaker. The cell density of the suspension cultures was determined by making use of a haemacytometer (Neubauer), with 0.4% trypan blue (Sigma) in 1 × PBS serving to determine cell viability. Once cell cultures has attained a density of 2-2.4 × 10^6 cells/ml, they were sub cultured to a lower density of 0.2-0.5 × 10^6 cells/ml in a minimum volume of 50 ml.

2.2.16 Transfection of Sf9 cells

Sf9 cells were seeded in a 6-well plate (Nunc^{TM}) at a density of 1 × 10^6 cells/ 35 mm well. The cells were allowed to attach at 27ºC for at least 1 hr. Cells were transfected with bacmid DNA using Cellfectin (GibcoBrl, Life Technologies), a cationic liposome reagent optimised for the transfection of insect cells. Five µl newly isolated bacmid DNA was added to 100 µl Grace's Insect medium, not containing any supplements. A second solution containing 6 µl Cellfectin reagent in 100 µl Grace's Insect medium, without supplements, was also prepared. The two solutions were combined, mixed gently and incubated for 45 min at room temperature without any agitation. This step allows for the formation of lipid-DNA complexes. The attached Sf 9 cells were washed with 2 ml Grace's Insect Medium (No supplements). Eight hundred µl Grace's Insect medium was added to each tube containing lipid-DNA complexes and mixed gently. The wash medium was removed and 1 ml of the diluted DNA-lipid complexes placed on the cells in each well. Cells were incubated at 27ºC for 5 hr. The transfection mixtures were then removed and replaced with 2 ml cell culture medium, containing FCS as well as antibiotics. The cells were incubated at 27ºC for 96 hr, after which the virus-containing supernatant was harvested and stored, protected from light, at 4ºC.

2.2.17 Infection of Sf9 cells

Sf9 cells were seeded in a 24-well plate (Nunc^{TM}) at a density of 0.3 × 10^6 cells/well. Cells were allowed to attach at 27°C for at least 1 hour and the insect cell medium removed. Two-hundred µl of the virus-containing supernatant harvested in section 2.2.16 and 300 µl of fresh insect cell medium were added into each well. Cells were incubated at 27°C for 72 hr and harvested with the medium by passing the cell suspension several times through a pipette tip. The harvested cells were centrifuged at 1500 rpm, washed twice with 1 × PBS-A and resuspended in 30 µl 1 × PBS-A. The samples were frozen way at -20°C.

2.2.18 Protein electrophoresis and Coomassie blue staining

Protein electrophoresis was performed in polyacrylamide gels under denaturing conditions, allowing separation of proteins based on their molecular sizes. The 0.75 mm thick 12% separating gel, containing 0.375 M Tris-HCl pH 8.8 and 0.1% SDS, and the 5% stacking gel, containing 0.125 M Tris-HCl pH 6.8 and 0.1% SDS, were both prepared from a stock solution of 30% acrylamide, 0.8% bisacrylamide. The gels were polymerised chemically by the addition of 0.008% (v/v) N,N,N',N' tetramethylethylene diamine (TEMED) and 0.08% (m/v) ammonium persulphate followed by incubation at 37°C for 20 min. Prior to electrophoretic analysis, the cell protein samples were mixed with an equal volume of 2 × Protein Solvent Buffer, PSB (125 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 10% β-mercaptoethanol, 20% glycerol), denatured by incubation at 95°C for 5 min and sonicated. Proteins were separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Also loaded on the acrylamide gel was the Rainbow marker (Amersham) and appropriate controls. Electrophoresis performed in 1 × TGS buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS) using the mini-gel Hoefer electrophoresis system and applying a current of 120 V for approximately 4 hr. The polyacrylamide gel was subsequently stained in Coomassie Brilliant Blue staining solution (0.125% Coomassie blue, 50% methanol, 10% acetic acid) for 30 min at room temperature and destained in 5% ethanol, 5% acetic acid at room temperature overnight.

2.3 RESULTS

2.3.1 Construction of the recombinant transfer vectors, pFASTBAC-VP7-mutant 177-RVLAIERYLKD, pFASTBAC-VP7-mutant 177- ALDSWK and pFASTBAC-VP7-mutant 177- RVLAIERYLKD -ALDSWK.

The transfer vector plasmid, pFASTBAC-VP7-mutant 177, was constructed by Dr Francois Maree (2001). During this procedure six additional amino acids, K, L, S, R, V and D, were inserted between the two arginine (R) amino residues in positions 177 and 178 of the native AHSV-9 VP7 protein. The nucleotide sequences that were inserted are target sites for the restriction endonucleases *Hind* III, *Xba* I and *Sal* I. By utilising primers containing these specific sites on their 5' ends, epitope regions from the HIV-1 subtype C viral strain Du151 gp 160 gene (kindly donated by the National Institute of Virology (NIV)) were amplified via PCR and cloned into the pFASTBAC-VP7-mutant 177 vector. The epitopes ALDSWK and RVLAIERYLKD were cloned individually into VP7, as depicted in Figure 2.3. After the cloning of the RVLAIERYLKD epitope region into the pFASTBAC-VP7-mutant 177 vector had been confirmed, the ALDSWK epitope region was cloned adjacent to RVLAIERYLKD. This was accomplished by following the same strategy used to clone the individual epitopes (Fig 2.3).

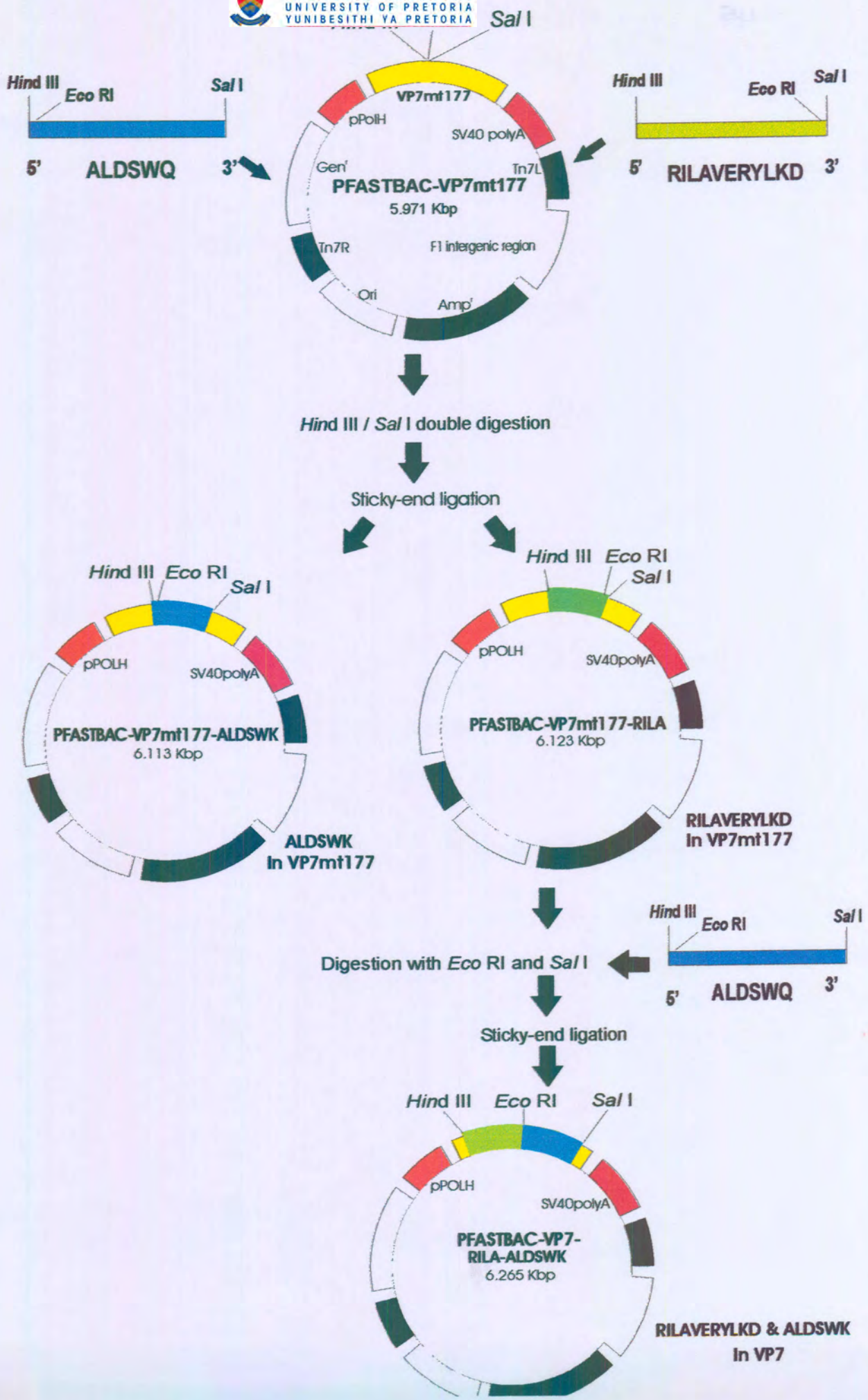

Fig 2.3 Schematic diagram of the protocol followed to generate the recombinant pFASTBAC vectors, pFASTBAC-VP7-mutant 177- RVLAIERYLKD, pFASTBAC-VP7-mutant 177- ALDSWK and pFASTBAC-VP7-mutant 177- RVLAIERYLKD -ALDSWK . In the first of this two-stage process, the single recombinant vectors, pFASTBAC-VP7-mutant 177- RVLAIERYLKD, pFASTBAC-VP7-mutant 177- ALDSWK, were constructed. The ALDSWK and RVLAIERYLKD sequence containing PCR products, as well as the pFASTBAC-VP7-mutant 177 vector were sequentially digested with restriction enzymes *Hind* III and *Sal* I. Following the cloning of the insert DNA into the vector molecule (ligation), the presence of the single recombinant pFASTBAC vectors was confirmed. The second stage of the process involved the construction of the dual recombinant, pFASTBAC-VP7-mutant 177- RVLAIERYLKD-ALDSWK, utilising a strategy similar to that outlined above. The ALDSWK- containing PCR product and the pFASTBAC-VP7-mutant 177- RVLAIERYLKD plasmid were each digested with restriction enzymes *Sal* I and *Eco* RI. Following the ligation of the insert and vector DNA recombinant pFASTBAC vectors were identified.

2.3.2 Identification of a suitable cloning site

Transfer vector pFASTBAC-VP7 mutant (mt) 177 was obtained from Dr F. Maree. There was some uncertainty over the precise nucleotide sequence inserted between nucleotides 548 and 549 of the native AHSV-9 VP7 gene during the construction of the transfer vector (Maree, 2000). Also unknown was the sequence of the nucleotides located immediately adjacent to the 3' end of the VP7 gene. Consequently, in order to solve these unknowns, and identify a suitable cloning site, the transfer vector was partially sequenced and these results confirmed by restriction enzyme digestion.

Sequencing of the multiple cloning site, inserted between nucleotides 548 and 549 of the VP7 gene, was accomplished by utilising the forward primer pol HF (Table 2.1). However, as this primer binds in the pFASTBAC polyhedron promoter region (3997-4041 bp), approximately 1201 bp upstream from the 3' end of the VP7 mt 177 gene, it could not be used to sequence the region downstream from the 3' end of the gene. The reverse primer SV40, which binds in the pFASTBAC SV40 poly A region (4413-4430 bp) (located downstream from the VP7 mt 177 gene) was used to obtain this sequence. Both sequencing reactions (section 2.2.4) were repeated in order to verify the sequences obtained in each case.

Five hundred and sixty base pairs and 664 bp of sequence data were obtained with primers pol HF and SV40, respectively (Figures 2.4 and 2.5). The sequence, obtained with forward primer pol HF and depicted in Figure 2.4, started with nucleotide 67 of the VP7 mt 177 gene and included the multiple cloning site (549-566 bp) within this gene. This data shows the order of the restriction enzyme recognition sites, inserted between nucleotides 548 and 549 bp in the native VP7 gene, to be 5' *Hind* III, *Xba I* and *Sal* I, and not 5' *Sal* I, *Xba I* and *Hind* III as previously suggested (Maree, 2000). Accordingly, the amino acids inserted into the AHSV-9 VP7 protein were in the order N terminal K, L, S, R ,V and D.

The sequence data obtained with reverse primer SV40 included nucleotide 846 of the native VP7 gene (highlighted) and all subsequent downstream sequence data up to nucleotide 4410 of the original pFASTBAC vector (Fig 2.5). The restriction endonuclease target sequences, in the region adjacent to the 3' end of the VP7 gene, were identified. A

second *Xba I* site in the pFASTBAC-VP7 mt 177 vector was located 31 bp from the 3' end of the VP7 mt 177 gene. Also of note is the presence of a modified *Hind* III site (Maree, 2000), located 62 bp from the 3' end of the VP7 mt 177 gene.

In order to confirm the sequencing results, the transfer vector was digested with restriction enzymes *Hind* III, *Xba I, Sal* I and *Eco* RI as described in section 2.2.7 (Fig 2.6). A single *Hind* III recognition site was present in the pFASTBAC-VP7 mt 177 vector, as indicated by the linearised plasmid band of 5950 bp (Fig 2.6, lane 3). As expected, the *Hind* III recognition site, present in the MCS in the VP7 mt 177 gene (549-554 bp), is unique in the vector. A *Hind* III site originally present in the pFASTBAC vector was lost during the cloning of the VP7 mt 177 gene (Maree, 2000). *Sal* I, recognising a single target sequence in the VP7 mt 177 gene (561-566), linearised the pFASTBAC-VP7 mt 177 vector (Fig 2.6, lane 5). It can be concluded that the *Sal* I recognition site is also unique in the vector. Digestion of the vector with *Xba* I yielded two DNA fragments of sizes 5291 bp and 659 bp (Fig 2.6, lane 4). This result was expected as the sequencing results showed two *Xba* I recognition sites in the vector- one in the inserted MCS and the other in the sequence downstream from the 3' end of the VP7 gene (Figures 2.4 and 2.5, respectively). As expected, *Eco* RI was not able to digest the plasmid (Fig 2.6, lane 6). The VP7 mt 177 gene does not contain an *Eco* RI recognition sequence and the single *Eco* RI site in the pFASTBAC plasmid was removed during the cloning of the VP7 mt 177 gene (Fig 2.5).

These restriction enzyme results confirm those of the sequencing data obtained. The *Hind* III and *Sal* I recognition sites present in the MCS in the pFASTBAC-VP7 mt 177 vector are unique, while *Xba* I has two target sites. The vector contains no recognition sequence for the restriction enzyme *Eco* RI. Consequently, *Hind* III and *Sal* I are candidate enzymes suitable for use in the initial cloning of inserts into the vector, while the *Eco* RI enzyme could be utilised for further cloning procedures.

Fig 2.4 The partial nucleotide sequence of the VP7 mt 177 gene (67 bp-625 bp) and the predicted partial amino acid sequence of the translation product. Indicated is the multiple cloning site (MCS) inserted between nucleotides 548 and 549 of the native VP7 gene and the additional amino acids (highlighted in black) encoded by this MCS.

Fig 2.5 The partial nucleotide sequence of the VP7 mt 177 gene and all subsequent bases up to nucleotide 4410 of the original FASTBAC vector. Lying immediately adjacent to the VP7 gene sequence (highlighted in black) is the sequence that was cloned during the construction of the pFASTBAC-VP7 mt 177 vector. Downstream from, and including the *Xba* I target site, is the nucleotide sequence from the original pFASTBAC vector. The *Hind* III site in this sequence was modified during the construction of the pFASTBAC-VP7 mt 177 plasmid.

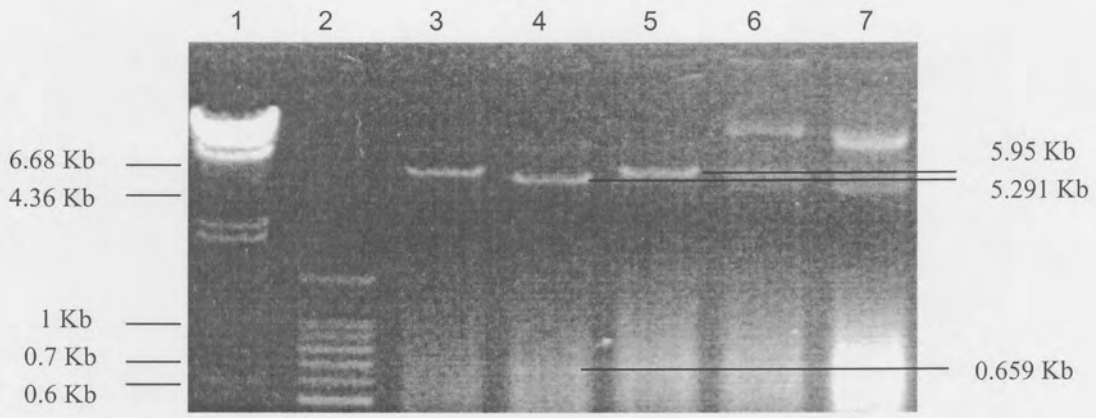

Fig 2.6 Agarose gel analysis of a single clone of the pFASTBAC-VP7 mt 177 vector, digested with restriction enzymes *Hind* III, *Xba I*, *Sal* I and *Eco* RI. Lanes 1 and 2 contain the DNA molecular weight markers λII and 100 bp ladder, respectively. Relevant sizes are indicated in Kb. A single band of 5.95 Kb is observed in lanes 3 and 5 containing vector DNA digested with enzymes *Hind* III and *Sal* I, respectively. Digestion of the vector with *Xba I* resulted in the formation of two bands 5.291 Kb and 0.659 Kb in size (lane 4). The three bands observed in lane 6, containing vector digested with enzyme *Eco* RI, look similar to those in lane 7, containing undigested vector plasmid DNA.

2.3.3 Amplification of the HIV-1 subtype C strain Du 151 epitope regions

Plasmid pFASTBAC-gp160, containing the HIV-1 subtype C Du 151 gp160 sequence, was kindly provided by Sibusiso Nkosi (NIV). Specific primer pairs were designed in order to amplify the RVLAIERYLKD and ALDSWK nucleotide sequences from the pFASTBAC-gp 160 template DNA. The primers designed are described in Table 2.3 and had several unique characteristics. Each primer had a G+C content of approximately 50%. Secondly, as the trimeric structure of the VP7 crystalline particles would not be able to tolerate an insertion of greater than 100 amino acids (Maree, 2000), the primers were designed to ensure that each epitope region amplified would not exceed 50 amino acids in size. The sequence amplified by the RILA primer pair was 152 bp in size and encoded 50 amino acids, while the sequence amplified with the ALD-FW and ALD-RV primers was 143 bp in size and encoded 47 amino acids (Fig 2.7). Thirdly, specific restriction endonuclease recognition sequences were added onto the 5' ends of each of the primers (Fig 2.7). This was to facilitate the cloning of the RVLAIERYLKD and ALDSWK nucleotide sequences into the vector, individually and in combination with each other. A *Hind* III enzyme recognition site was added onto the 5' end of the RILA-FW primer, while *Eco* RI and *Sal* I sites were added onto the 5' end of RILA-RV. Recognition sites for restriction enzymes *Hind* III and *Eco* RI were added onto the 5' end of ALD-FW and a *Sal* I recognition site was placed onto the 5' end of ALD-RV. In addition, three cytosine (C) bases were added onto each of the primers to increase the efficiency of restriction enzyme digestion at the ends of the PCR products. The inclusion of these additional nucleotides into the design of the primers resulted in the amplification of larger PCR products. The amplification product containing the RVLAIERYLKD region was 176 bp in size, while that containing the ALDSWK region was 167 bp (Fig 2.7).

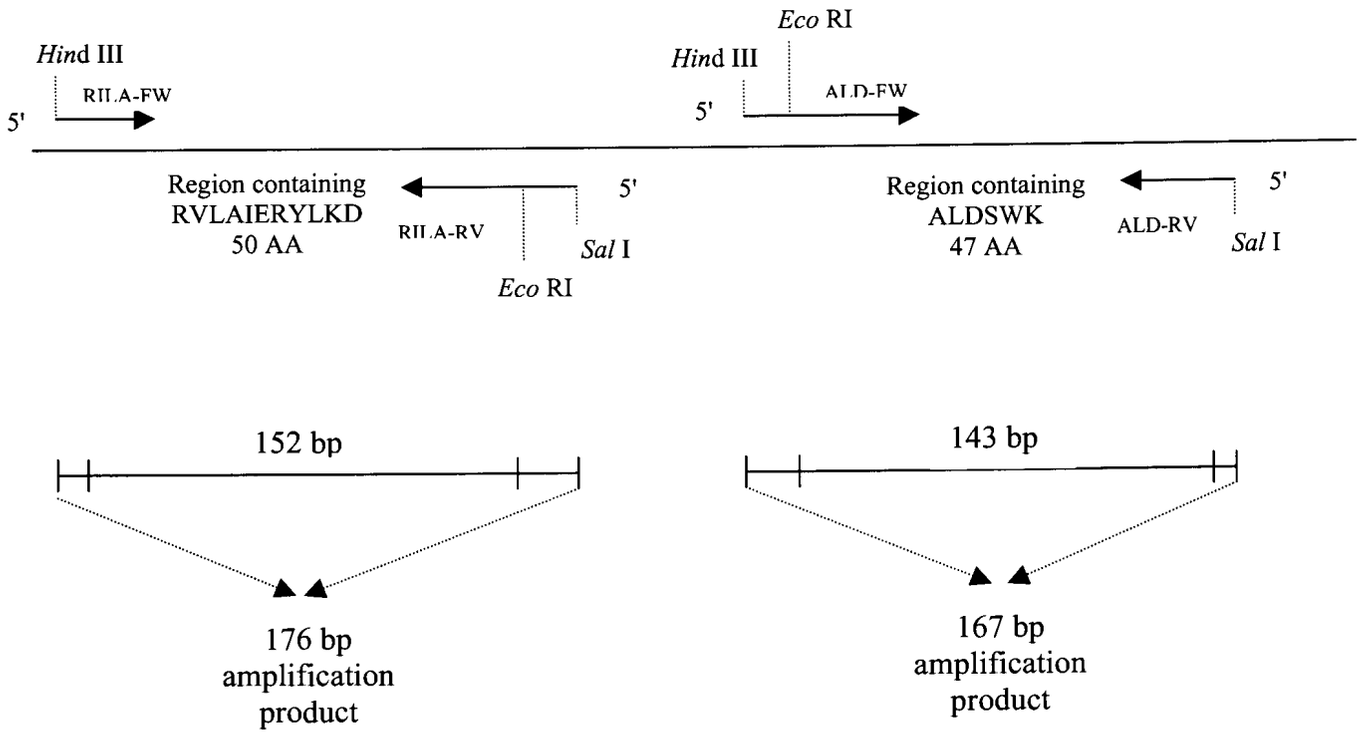

Fig 2.7 Schematic diagram of the primers used to amplify the nucleotide sequences, containing the RVLAIERYLKD and ALDSWK regions, from gp160 template DNA. Due to size constraints of the VP7 presentation proteins, sequences amplified could not exceed a combined 100 amino acids when translated. The sequences encoding the RVLAIERYLKD and ALDSWK regions are thus 152 bp and 143 bp in size, respectively. However, due to the presence of additional restriction enzyme sites present on the 5' ends of the primers (added in order to facilitate cloning into the vector), amplification products of 176 bp and 167 bp result.

PCR reactions utilising these primers were performed as described in section 2.2.6. and the PCR products were visualised on a 1% agarose gel (Fig 2.8). The 176 bp PCR product, containing the RVLAIERYLKD-encoding sequence, as well as the 167 bp PCR product, containing the ALDSWK-encoding sequence, were visualised in lanes 4 and 3, respectively. The reaction amplifying the RVLAIERYLKD region was more successful, resulting in a more intense DNA band. The absence of bands in the lane containing the negative control reaction (lane 5) indicates that no foreign DNA contamination occurred while the PCR reactions were being prepared.

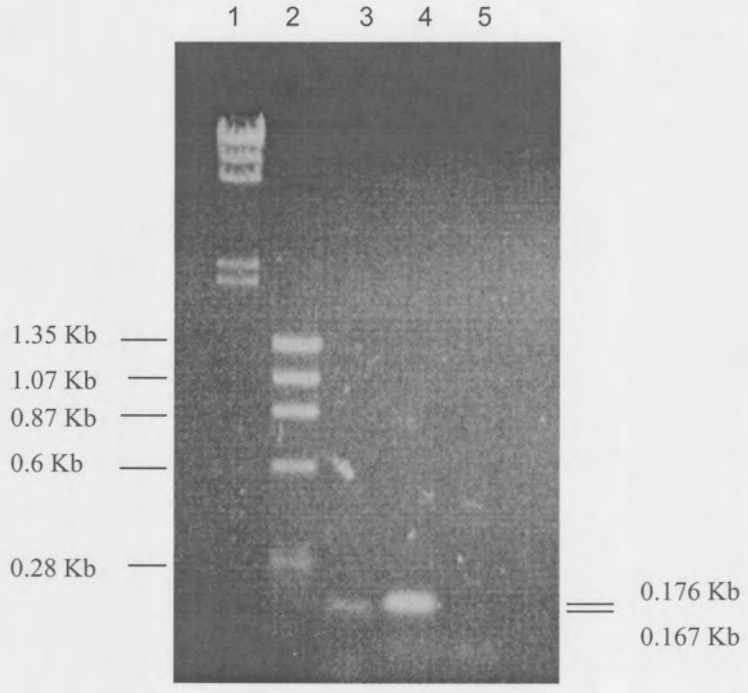

Fig 2.8 Agarose gel electrophoresis analysis of PCR products containing the RVLAIERYLKD and ALDSWK-encoding sequences. Lanes 1 and 2 contain the DNA molecular weight markers λII and ϕX, respectively. Relevant sizes are indicated in Kb. A 0.167 Kb amplification product, containing the ALDSWK sequence is visualised in lane 3, while a 0.176 Kb PCR product, containing the RVLAIERYLKD sequence, is observed in lane 4. Lane 5 contains the contents of the negative control reaction.

2.3.4 Restriction enzyme digestion of PCR products and the pFASTBAC-VP7 mt 177 vector

Prior to the ligation procedure, the insert and vector DNA must be digested with restriction endonucleases that produce complementary ends in these molecules.

The PCR products, containing the sequences ALDSWK and RVLAIERYLKD, as well as the pFASTBAC-VP7 mt 177 vector, were sequentially digested with restriction endonucleases *Hind* III and *Sal* I. Reactions were performed and the digestion products purified as described in section 2.2.7. The DNA concentration was estimated following 1% agarose gel electrophoresis. As digestion with restriction enzymes *Hind* III and *Sal* I results in the formation of non-complementary sticky ends, there is no need to utilise the standard procedure of vector dephosphorylation. Should digestion with the two enzymes be complete, the vector will not be able to self-ligate during the ligation reactions.

The PCR products and the pFASTBAC-VP7 mt 177 vector, digested with endonucleases *Hind* III and *Sal* I and following purification, are shown in Figure 2.9. The intensity of the resultant bands indicate that the concentration of the digested vector (lane 5) is approximately 1/4 that of the RVLAIERYLKD PCR product (lane 4) and approximately 1/2 of the concentration of the ALDSWK PCR product (lane 3). It is important to note the concentrations of the insert and vector DNA molecules prior to the preparation of a ligation reaction as ligation reactions are prepared in a specific vector:insert ratio.

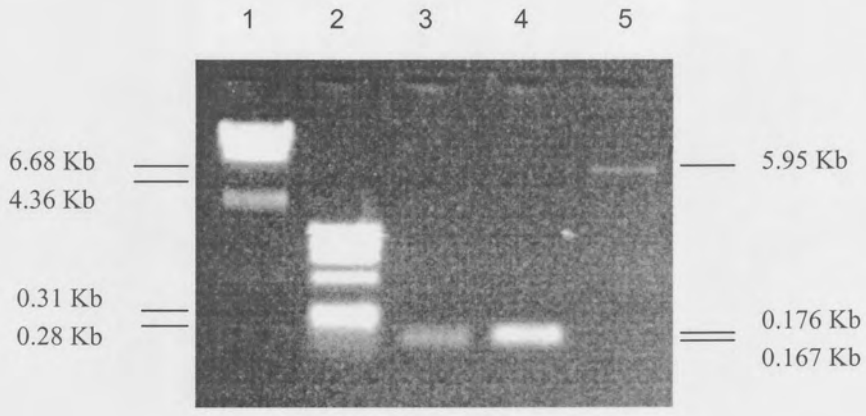

Fig 2.9 Agarose gel analysis of the RVLAIERYLKD and ALDSWK-containing PCR products and the pFASTBAC-VP7 mt 177 vector, all digested sequentially with the restriction enzymes *Hind* III and *Sal* I and purified by the High pure PCR purification kit. Lanes 1 and 2 contain the DNA molecular weight markers λII and ϕX, respectively. Relevant sizes are indicated in Kb. The digested PCR products, containing the ALDSWK (0.167 Kb) and RVLAIERYLKD (0.176 Kb) sequences, can be visualised in lanes 3 and 4, respectively. The linearised pFASTBAC-VP7 mt 177 vector (5. 95 Kb) can be observed in lane 5.

2.3.5 Ligation of the insert and vector DNA and transformation into competent bacterial cells.

Three ligation reactions were prepared as described in section 2.2.8. Two of the reactions contained PCR products encoding the ALDSWK/RVLAIERYLKD epitopes and the linearised pFASTBAC-VP7 mt 177 vector in an insert-vector ratio of 3:1. The control reaction was prepared to ascertain whether digestion of the vector DNA with the restriction endonucleases *Hind* III and *Sal* I had been complete (section 2.3.4). Ligation success was determined by transforming the ligation reactions into XL-1 Blue cells, according to the protocol in section 2.2.2. Each transformation reaction was plated out onto selective $Tet^{+}Amp^{+}$ agar plates and incubated overnight at 37°C.
Bacterial colonies were observed following transformation. However, no bacterial colonies were observed on the negative control plate onto which cells, transformed with vector DNA ligation reactions, were plated out. This indicates that digestion of the vector with restriction enzymes *Hind* III and *Sal* I was complete and the non-complementary sticky ends formed were not able to religate during the ligation reaction. It was concluded that the ligation reactions were successful and the colonies observed contained recombinant vector. This theory was subsequently tested by means of the colony dot blot procedure.

2.3.6 Colony blot for identification of recombinant clones

The bacterial colonies were screened for the presence of recombinant plasmid by means of a colony blot procedure. This method, described in section 2.2.10, involves the *in situ* hybridisation of DIG-labelled probes to the DNA of bacterial clones.

By comparing the colour intensities of the DIG-labelled probe dilutions with those of the DIG-labelled control DNA (Figure 2.10), the concentrations of the ALDSWK and RVLAIERYLKD DIG-labelled probes were each calculated to be 1 ng/µl. The results obtained with the DIG-labelled probe are shown in Figure 2.11. The bacterial DNA believed to be plasmid pFASTBAC-VP7 mt 177-RVLAIERYLKD (on right) hybridised with the DIG-labelled probe containing the RVLAIERYLKD sequence. All four colonies on the membrane yielded a positive signal and displayed a blue-purple colour precipitate

after detection. The four colonies present on the original selective agar plate thus contained the recombinant vector into with the RVLAIERYLKD sequence.

The bacterial DNA from colonies believed to be harbouring recombinant plasmid pFASTBAC-VP7 mt 177-ALDSWK (left), did not hybridise to the ALDSWK DIG-labelled probe. There may be two explanations for this result. Either the colonies contained religated vector, which is unlikely as digestion of the vector DNA is believed to have been complete (section 2.3.5), or the labelled probe containing the ALDSWK sequence was not sufficiently denatured into single stranded form to allow for hybridisation. The latter explanation seems likely as the positive control included in the hybridisation reaction with the ALDSWK probe did not yield a blue-purple precipitate (results not shown). These colony blot results were further verified by means of PCR.

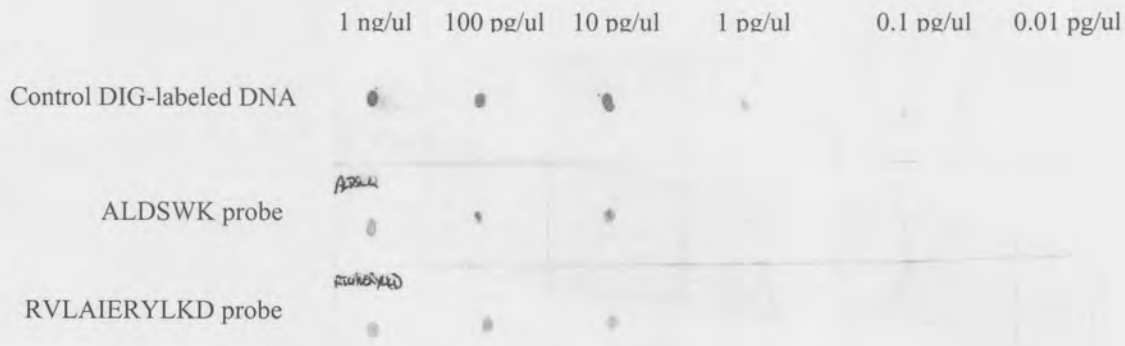

Fig 2.10 Determination of the yield of DIG-labelled probes by means of the direct detection method. Serial dilutions of the DIG-labelled control DNA (supplied in the kit), the DIG-labelled ALDSWK PCR product and the DIG-labelled RVLAIERYLKD product were dot-blotted onto a nylon membrane. Following a immunological detection procedure, the dot colour intensities that resulted from each of the PCR product dilutions were compared to those of the defined dilutions of Dig-labelled control DNA. Taking the dilution factors in account, the concentrations of the DIG-labelled PCR probes were calculated.

Fig 2.11 Results from the colony blot procedure used to identify recombinant bacterial clones. The colonies imprinted onto the surface of the nylon membrane on the right yielded a positive blue-purple signal following hybridisation with the RVLAIERYLKD Dig-labelled probe and immunological detection. The colonies imprinted onto the membrane on the left however did not yield the same positive result when hybridised with the ALDSWK DIG-labelled probe. The blotches of discoloration observed are the remnants of the bacterial colonies that were lifted onto the membrane and not a positive hybridisation signal.

2.3.7 PCR verification of recombinant clones

PCR was used to verify the results of the colony blot procedure (section 2.3.6). Plasmid DNA was isolated from each of the bacterial clones in question and added into PCR reactions containing the primers VP7FWD1 and VP7RVH1 (section 2.2.11). These primers, specific for VP7, were expected to yield PCR products of specific sizes depending on whether or not the plasmid DNA templates were recombinant. A positive control PCR reaction, containing non-recombinant pFASTBAC-VP7 mt 177 vector DNA, and a negative control reaction, containing no template DNA were also prepared. The resulting PCR products were analysed on an agarose gel after 1% agarose gel electrophoresis.

Primers VP7FWD1 and VP7RVH1 amplified a DNA fragment of approximately 529 bp from all of the plasmid templates (Fig 2.12, lanes 3-10). This indicated that all the clones tested did indeed contain recombinant plasmid and not religated vector. The presence of religated vector would have resulted in the amplification of a 358 bp band, as observed in the positive control reaction (lane 11). It was concluded that the lack of positive identification by the colony blot hybridisation was probably because the ALDSWK-specific probe had not been sufficiently denatured.

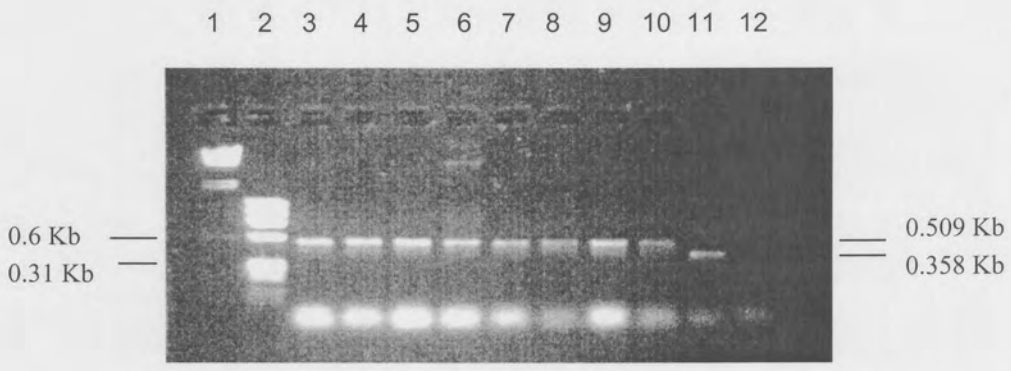

Fig 2.12 Agarose gel analysis of PCR products amplified with primers VP7FWD1 and VP7RVH1 from various candidate recombinant clones. Lanes 1 and 2 contain the DNA molecular weight markers λII and ϕX, respectively. Relevant sizes are indicated in Kb. A DNA fragment of 0.509 Kb was amplified from all the candidate plasmids (Lanes 3-10). The positive control reaction, containing the pFASTBAC-VP7 mt 177 vector template, yielded a band of 0.358 Kb (Lane 11). No bands were amplified in the negative control reaction (Lane 12). The conspicuous presence the intense bands approximately 100 bp in size indicate that certain PCR components, such as primers or nucleotides, were in excess in the PCR reactions.

2.3.8 Partial sequencing of recombinant plasmids pFASTBAC-VP7 mt 177-RVLAIERYLKD and pFASTBAC-VP7 mt 177- ALDSWK

Partial sequencing of the recombinant plasmids pFASTBAC-VP7 mt 177-RVLAIERYLKD and pFASTBAC-VP7 mt 177-ALDSWK was performed to confirm the correct insertion of the epitope regions. Sequencing reactions were performed on two randomly chosen plasmids, utilising the forward primer VP7FWD1, as described in section 2.2.4.

The partial sequences of the template plasmids pFASTBAC-VP7 mt 177-RVLAIERYLKD and pFASTBAC-VP7 mt 177-ALDSWK are shown in Figures 2.13 and 2.14, respectively. Translation indicated that no frame shift mutations occurred during the insertion of the epitope regions into the vector. The amino acids encoded upstream, as well as the amino acids downstream from the insertion site, were unchanged. The inserted amino acids were identical in sequence to the amino acids of the epitope regions in the native gp 160 protein, depicted in Fig 2.2.

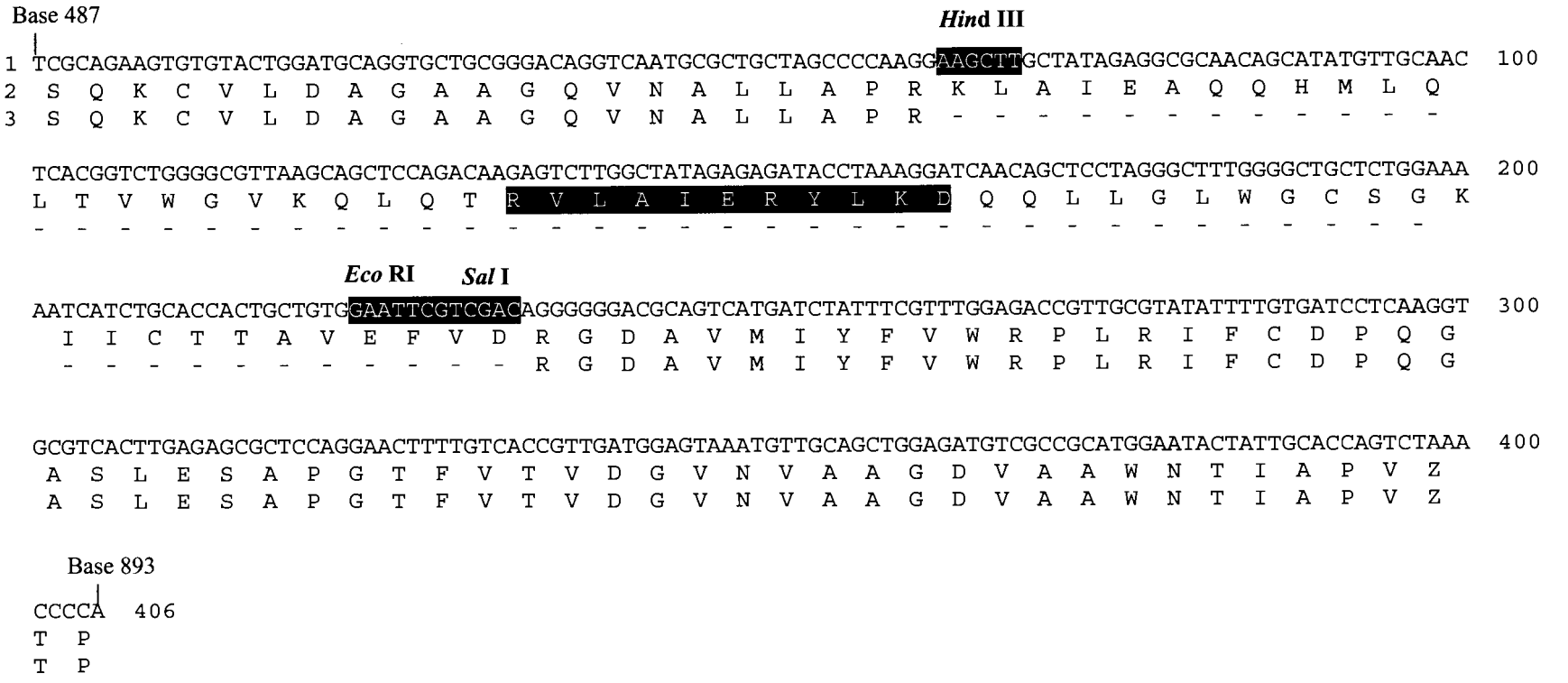

Fig 2.13 The partial nucleotide sequence of the VP7 mt 177- RVLAIERYLKD gene (487bp - 893bp) and the predicted partial amino acid sequence of the translation product (rows 1 and 2, respectively). Highlighted in black is the RVLAIERYLKD amino acid sequence as well as the relevant restriction enzyme sites. Row 3 contains the corresponding amino acid sequence of the native VP7 protein. Dashes indicate where the amino acids of the epitope equivalent RVLAIERYLKD region were inserted into the native VP7 protein. No frameshift mutations occurred during the insertion of the additional amino acids, as the amino acids upstream and downstream from the insertion site are identical to those of the native VP7 protein.

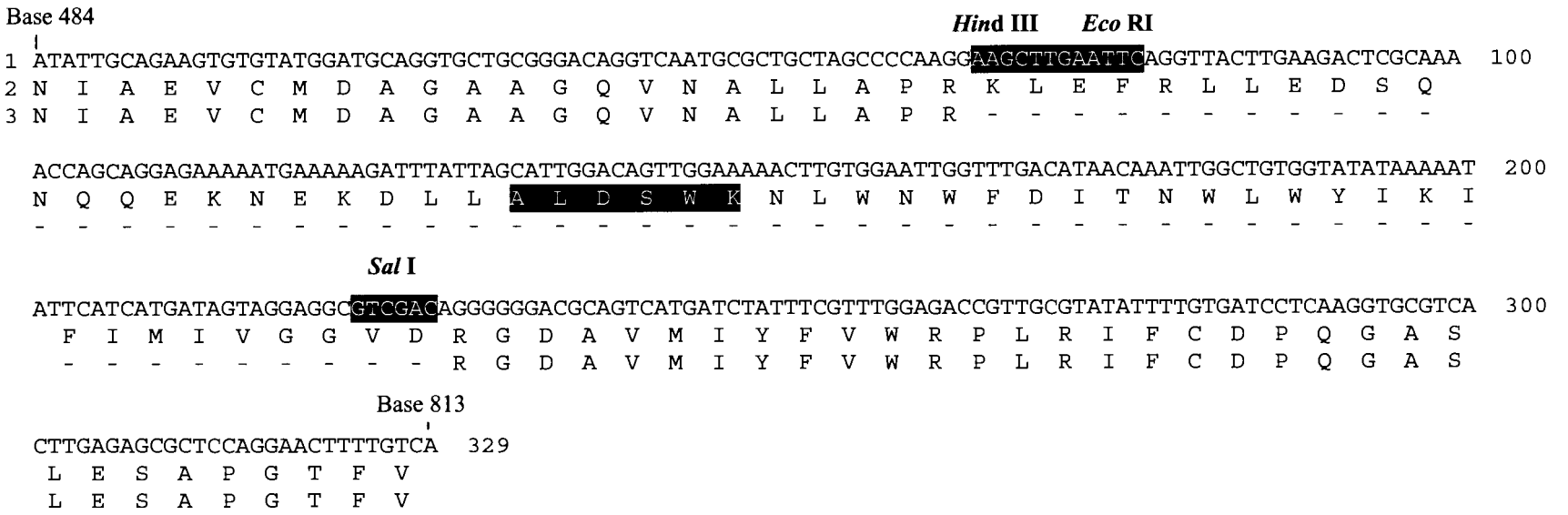

Fig 2.14 The partial nucleotide sequence of the VP7 mt 177- ALDSWK gene (484 bp-813 bp) and the predicted partial amino acid sequence of the translation product (rows 1 and 2, respectively). Highlighted in black is the ALDSWK amino acid sequence and the relevant restriction enzyme sites. Row 3 contains the corresponding amino acid sequence of the native VP7 gene. Dashes indicate between which amino acids of the native VP7 protein the epitope-equivalnet region ALDSWK is inserted. No frameshift mutations occurred during the insertion of the additional amino acids as the amino acids upstream as well as downstream from the insertion site are identical to those of the native VP7 protein.

2.3.9 Construction of the recombinant transfer vector pFASTBAC-VP7 mt 177 RVLAIERYLKD- ALDSWK

The epitopes RVLAIERYLKD and ALDSWK have been cloned individually into the pFASTBAC-VP7 mt 177 vector, with the aim of being presented by the AHSV-9 VP7 protein. However, a stronger antibody-mediated immune response may be induced by the presentation of both these regions on the surface of the VP7 protein. The construction of the recombinant transfer vector pFASTBAC-VP7 mt 177-RVLAIERYLKD- ALDSWK was thus attempted.

The strategy followed to construct this dual recombinant vector is shown in Fig 2.3 and similar to that outlined when constructing the single recombinant vectors (sections 2.3.4 and 2.3.5). Both the PCR product containing the ALDSWK-encoding sequence and the recombinant pFASTBAC-VP7 mt 177- RVLAIERYLKD vector were digested with restriction enzymes *Sal* I and *Eco* RI. The ALDSWK PCR product and the pFASTBAC-VP7 mt 177- RVLAIERYLKD vector were then ligated in the recommended 3:1 insert\vector ratio. Transformation reactions were plated out onto selective Tet^+ Amp^+ agar plates and incubated overnight at 37°C.

Bacterial colonies, transformed with ALDSWK/pFASTBAC-VP7 mt 177- RVLAIERYLKD ligation reaction, were visualised on test plates. Also observed were colonies transformed with the vector DNA control reaction. This indicated that the vector was able to self-ligate and recirculize during the ligation reaction. It was concluded that digestion of the pFASTBAC-VP7 mt 177- RVLAIERYLKD vector with restriction enzymes *Sal* I and *Eco* RI was incomplete. As the vector was able to religate, the colonies observed on the test plates may have contained either recombinant or religated vector.

2.3.10 Identification of recombinant clones through restriction enzyme analysis

In order to determine which clones were dual recombinants, containing both the RVLAIERYLKD and ALDSWK sequences, the plasmids were digested with specific restriction endonucleases.

Plasmid DNA from each of the clones was isolated and sequentially digested with restriction enzymes *Hind* III and *Sal* I. The recognition sites for the *Hind* III and *Sal* I enzymes lie on either side of the MCS in the VP7 mutant 177 gene into which the epitope sequences were inserted. By evaluating the sizes of the DNA fragments resulting from digestion, it was possible to determine whether both the epitope sequences were present in the VP7 mt 177 gene. Reactions were performed as described in section 2.2.7. As contaminating RNA would obscure DNA bands of 100-300 bp, treatment with the Rnase A enzyme (Roche diagnostics) facilitated the digestion of the RNA and visualisation of the expected bands following 1% agarose gel electrophoresis.

The results indicated that most of the randomly selected bacterial clones did contain a dual recombinant vector. The expected 331 bp fragment, containing the RVLAIERYLKD and ALDSWK sequences, was obtained from certain restriction enzyme reactions (Fig 2.15, lanes 3,4,6,8). A religated vector, containing only the RVLAIERYLKD sequence, would have yielded a band of 171 bp. The two additional digestion products, approximately 1.2 Kb and 0.9 Kb in size, observed in lane 5 were unexpected. This clone was deemed undesirable and subsequently discarded.

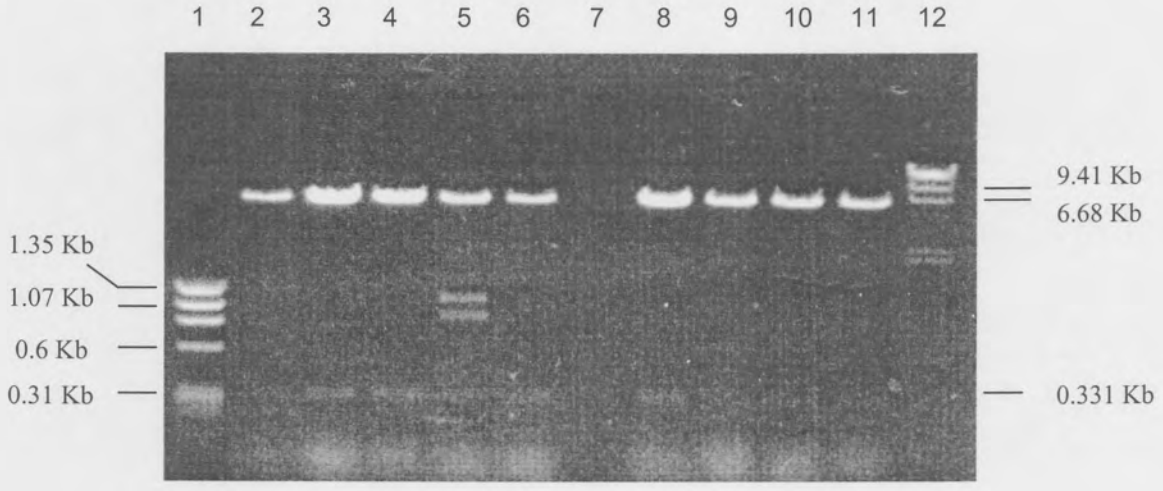

Fig 2.15 Agarose gel analysis of candidate dual recombinant plasmids after sequential digestion with restriction enzymes *Hin*d III and *Sal* I. Lanes 1 and 12 contain the DNA molecular weight markers ϕX and λII, respectively. The relevant sizes are indicated in Kb. Fragments 0.331 Kb and 5.962 Kb in size were yielded from each of the digested candidate plasmid (Lanes 2-6, Lanes 8-11). Two additional bands of sizes 1.2 Kb and 1 Kb were observed in lane 5. No sample was loaded in lane 7.

2.3.11 Confirmation of dual recombinants with PCR

To confirm the previous results, PCR reactions were performed utilising the primer pair VP7FWD1 and VP7RVH1 (section 2.2.11). These primers were designed to bind on either side of the MCS (549-566 bp), present in the VP7 mt 177 gene (Table 2.4). Two positive control reactions, containing plasmids pFASTBAC-VP7 mt 177 and pFASTBAC-VP7 mt 177- RVLAIERYLKD, were prepared as was a negative control reaction containing no DNA. The resulting products were separated by 1% agarose gel electrophoresis.

An amplification product of 673 bp was observed in lanes 3 and 6 of Fig 2.16. This indicated that the template DNA included in these PCR reactions contained both the RVLAIERYLKD and ALDSWK sequences. These clones were thus dual recombinants, containing both epitopes in the VP7 mt 177 gene. None of the amplification products observed in lanes 4 and 5 were the same size as the 358 bp band amplified from the vector plasmid (lane 7) or the 529 bp product amplified from the single recombinant plasmid (lane 8). It was concluded that the template DNA in these reactions did not contain the VP7 gene, the VP7 gene with the RVLAIERYLKD sequence or the VP7 gene with both the RVLAIERYLKD and ALDSWK sequences. These clones were not analysed further and discarded. The absence of bands in lane 9, which contains the negative control reaction, indicates that no contamination occurred during the preparation of the PCR reactions. In order to remove the element of primer non-specific binding and subsequent amplification of non-target sequences, as observed in lanes 3-6, it would be recommended to increase the primer annealing temperature of the PCR reactions.

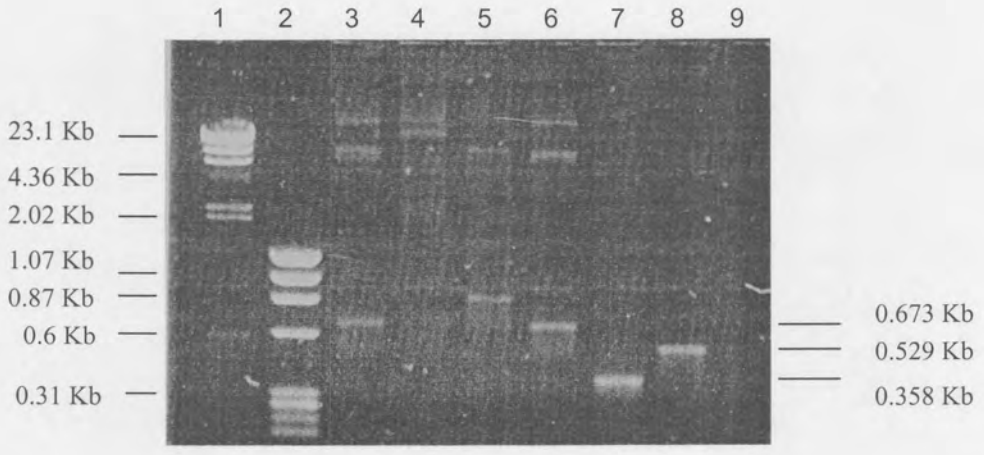

Fig 2.16 Agarose gel analysis of the PCR products amplified with primers VP7FWD1 and VP7RVH1 from several candidate dual recombinant clones. Lanes 1 and 2 contain the DNA molecular weight markers λII and ϕX, respectively. The relevant sizes are indicated in Kb. Lanes 3 and 6 contain the PCR products amplified from several candidate clone templates. Lane 8 contains the products amplified from the pFASTBAC-VP7 mt 177-RVLAIERYLKD DNA template (positive control), while lane 7 contains the PCR product amplified from the pFASTBAC-VP7 mt 177 vector DNA (2^{nd} positive control). No bands were visualised in lane 9, containing the negative control reaction.

2.3.12 Partial sequencing of the recombinant plasmid pFASTBAC-VP7 mt 177-RVLAIERYLKD- ALDSWK

Partial sequencing of the recombinant plasmid pFASTBAC-VP7 mt 177-RVLAIERYLKD- ALDSWK was performed to confirm the correct insertion of the ALDSWK sequence into the VP7 mt 177 gene. Sequencing reactions were performed on a randomly chosen clone utilising the forward primer VP7FWD1, as described in section 2.2.4

The partial sequence of plasmid pFASTBAC-VP7 mt 177- RVLAIERYLKD- ALDSWK is given in Fig 2.17. As observed, the ALDSWK sequence is situated adjacent to the RVLAIERYLKD sequence in the VP7 mt 177 gene. Upon translation of this nucleotide sequence, it can be concluded that no frame shift mutations occurred during the insertion of the ALDSWK sequences into the vector. The amino acids encoded upstream and downstream from the insertion site were unchanged. In addition, the amino acids that were inserted are identical in sequence to the amino acids of the ALDSWK containing region in the native gp160 protein depicted in Fig 2.2.

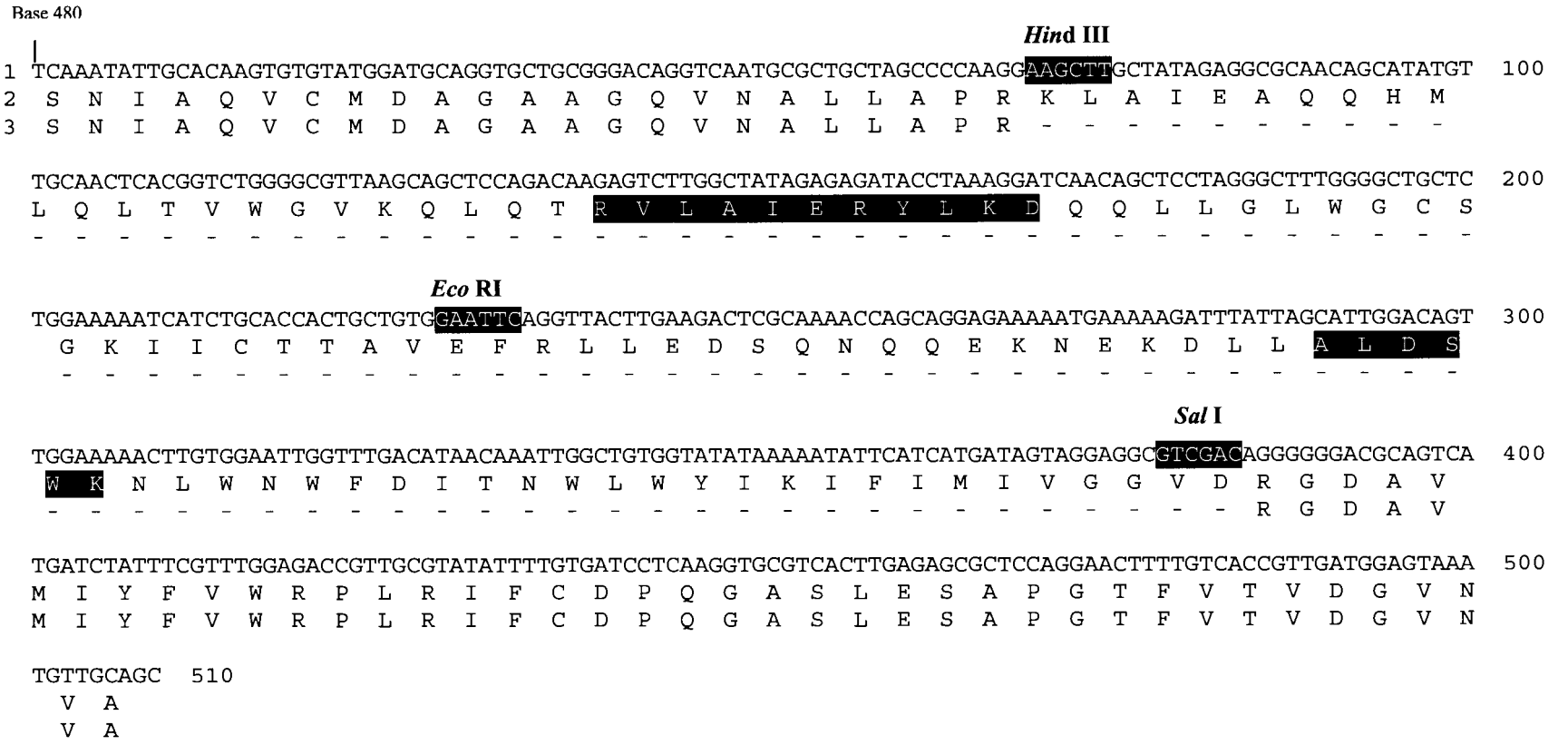

Fig 2.17 The partial sequence of the VP7 mt 177-RVLAIERYLKD-ALDSWK gene (480 bp- 990 bp) and the predicted partial amino acid sequence of the translation product (row 1 and 2, respectively). Highlighted in black are the RVLAIERYLKD and ALDSWK amino acid sequences as well as the relevant restriction enzyme sites. Row 3 contains the corresponding amino acid sequence of the native VP7 protein. Dashes indicate where the amino acids of the epitope-equivalent regions were inserted into the native VP7 protein. No frameshift mutations occurred during the insertion of the additional amino acids, as the amino acids upstream and downstream from the insertion site were identical to those of the native VP7 protein.

2.3.13 Generation of recombinant baculoviruses

Following the successful insertion of the epitope sequences into the pFASTBAC-VP7 mt 177 vector, recombinant baculoviruses were generated utilising the well-known Bac-to-Bac™ Baculovirus Expression system. The first stage in this process is the generation of recombinant bacmid DNA in *E. coli* DH10 Bac cells (Transposition). The presence of the foreign genes in the bacmid DNA is then confirmed and the recombinant DNA is used to transfect Sf9 insect cells for the generation of recombinant baculoviruses.

Thus plasmids pFASTBAC-VP7 mt 177- RVLAIERYLKD, pFASTBAC-VP7 mt 177-ALDSWK and pFASTBAC-VP7 mt 177- RVLAIERYLKD- ALDSWK were transformed into competent *E.coli* DH10 Bac cells, as described in section 2.2.13. Bacmid DNA was isolated from the potentially recombinant white bacterial colonies using a method used for the isolation of DNA upto several hundred Kb long (section 2.2.14). This protocol is an adaptation of the alkaline lysis method used to isolate plasmid DNA. As the bacmid DNA is so large (130 Kb), great care was taken during the isolation procedure to ensure that the DNA was not nicked. Samples were not vortexed and the isolated bacmid DNA was not mechanically resuspended to avoid shearing. As the bacmid DNA was to be transfected into insect cells, it was imperative that the DNA remained sterile. To this end all steps subsequent to the 70% ethanol wash step were performed in a sterile laminar flow hood. The DNA isolated was analysed electrophoretically in a 0.5% agarose gel in order to confirm the presence of high molecular weight bacmid DNA.

In order to confirm the presence of RVLAIERYLKD and ALDSWK epitope sequences in the bacmid DNA, PCR reactions containing the ALD- and RILA-specific primers were performed.

Utilising the sets of primers mentioned in Table 2.3, as well as primer pair RILA-FW and ALD-RV, PCR reactions were prepared as described in section 2.2.6. Primer pairs RILA-FW / RILA-RV and ALD-FW / ALD-RV were added into PCR reactions with template bacmid DNA believed to contain the RVLAIERYLKD and ALDSWK sequences, respectively. Primer pair RILA-FW and ALD-RV was added to the PCR reactions with bacmid thought to contain both these sequences. A negative control reaction was also

prepared to test for possible DNA contamination. The PCR amplification products were analysed electrophoretically on a 1% agarose gel.

PCR reactions containing the RILA-FW / RILA-RV primers yielded the expected 176 bp amplification band (Fig 2.18, lanes 2-4). Also expected was the 167 bp band amplified in the PCR reactions with primers ALD-FW / ALD-RV (Fig 2.18, lanes 8-11). In addition, primer pair RILA-FW / ALD-RV yielded the expected 419 bp PCR amplification product (Fig 2.18, lanes 5-7). No contamination with foreign DNA occurred, as no bands were amplified in the negative control reaction (Fig 2.18, lane 12). It can thus be concluded that each of the bacterial clones tested via PCR contained the RVLAIERYLKD sequence, the ALDSWK sequence or both these epitope sequences. The recombinant bacmid DNA was ready to be transfected into insect cells.

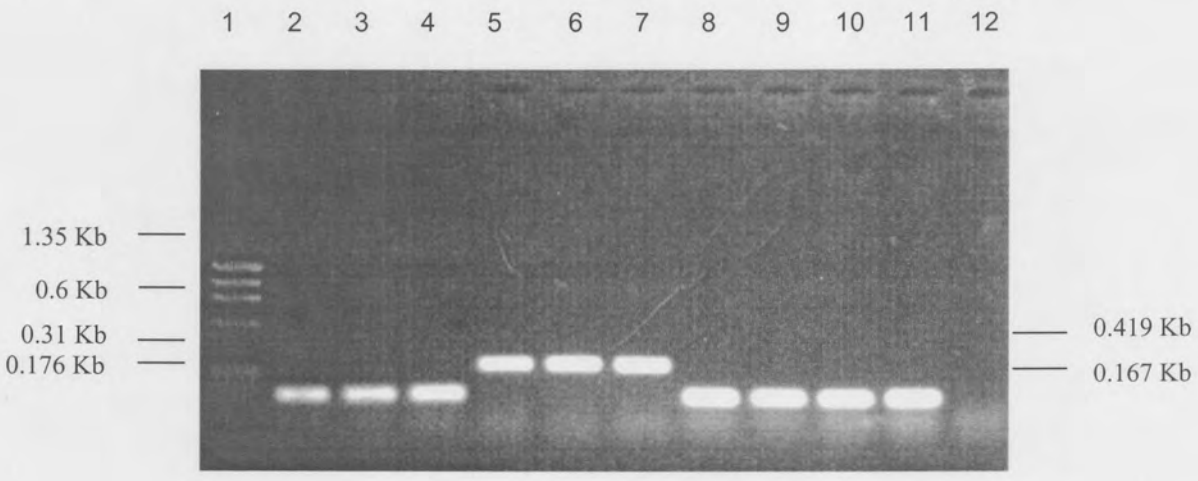

Fig 2.18 Agarose gel analysis of the PCR products amplified from several clones of each of the three recombinant plasmids pFASTBAC-VP7 mt 177-RVLAIERYLKD, pFASTBAC-VP7 mt 177-ALDSWK and pFASTBAC-VP7 mt 177-RVLAIERYLKD-ALDSWK. Lane 1 contains the DNA molecular weight marker ϕX. Lanes 2-4, contain the PCR products amplified from the pFASTBAC-VP7 mt 177-RVLAIERYLKD template DNA. Lanes 5-7 contain the PCR products amplified from the pFASTBAC-VP7 mt 177-RVLAIERYLKD-ALDSWK template DNA while lanes 8-11 contain the PCR products yielded after amplification from the pFASTBAC-VP7 mt 177-ALDSWK template. No bands were visible in the negative control (Lane 12).

The final step in generating recombinant baculoviruses, capable of replicating in insect cells, is the transfection of recombinant bacmid DNA into Sf9 insect cells using Cellfectin, a cationic lipid.

The protocol followed to transfect Sf9 insect cells with Cellfectin was described in section 2.2.16. Following the uptake of DNA by the insect cells, replication and transcription of the bacmid DNA takes place and after approximately 72 hrs, recombinant budded virus is obtained in the supernatant medium. One tenth of this virus-containing supernatant was used to infect new insect cells (as described in section 2.2.17) and the remainder stored at 4°C, protected from the light.

Approximately 3 days following the initial transfection of the bacmid DNA, cellular cytopathic effects became apparent, with the insect cells appearing swollen and becoming more easily detached from the monolayer than uninfected cells.

2.3.14 Expression of chimeric VP7 proteins in Sf9 insect cells

To confirm that baculoviruses capable of expressing chimeric VP7 protein were generated, infected Sf9 insect cells were harvested and their proteins analysed by SDS-PAGE and Coomassie brilliant blue staining.

Three days post-infection the infected cells were harvested as described in section 2.2.18. The proteins in these total cell lysates were resolved by SDS-PAGE and visualised by Coomassie brilliant blue staining (section 2.2.18). The chimeric VP7-ALDSWK protein band, approximately 47.3 KDa in size, is clearly visible in lane 3 of Fig 2.19. Visible in lane 4 is the VP7-RVLAIERYLKD protein band approximately 47.8 KDa in size, while lanes 5 and 6 contain the unique VP7- ALDSWK- RVLAIERYLKD protein (55.8 KDa).

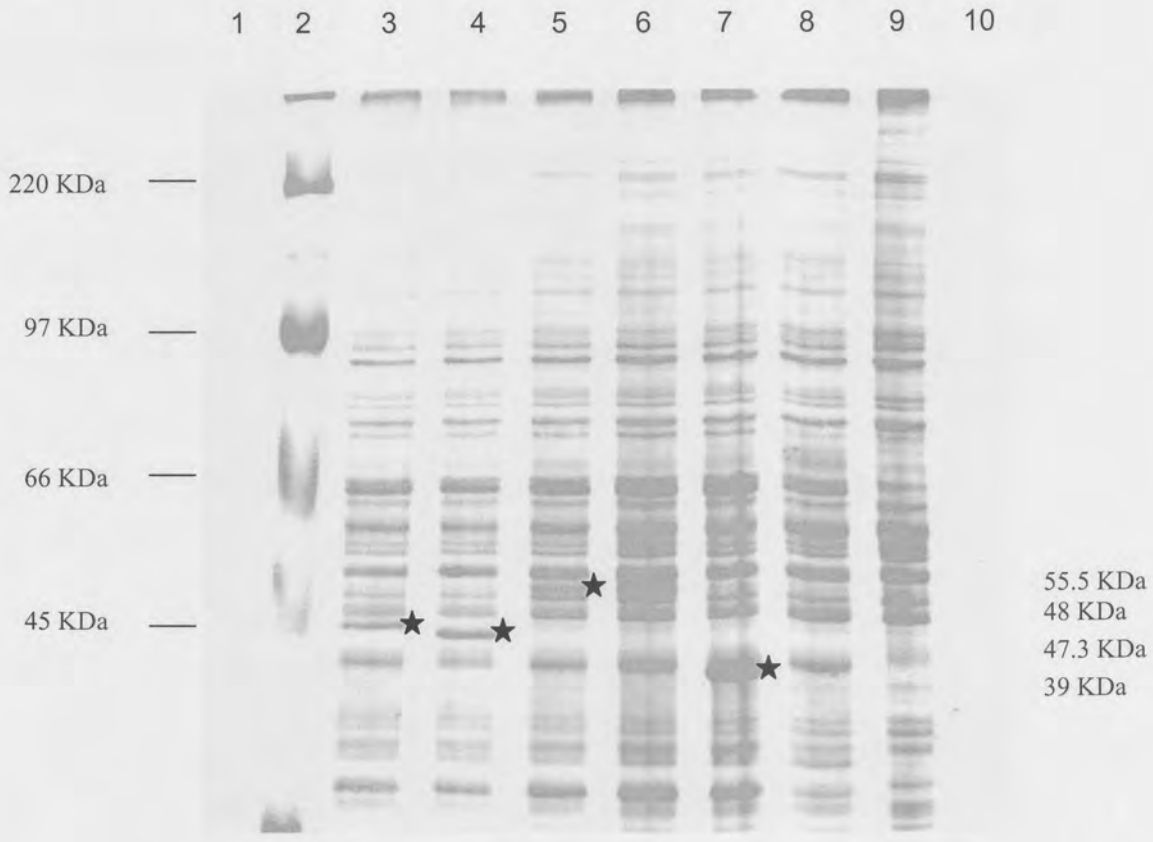

Fig 2.19 12 % SDS-PAGE analysis of cell lysates of insect cells either uninfected or infected with recombinant/wild type baculoviruses. Visualised in lane 2 is the Rainbow protein size marker (Amersham). The relevant sizes are indicated in KDa. The black stars are indicative of the unique proteins expressed. Lane 3 contains proteins isolated from insect cells infected with the VP7-ALDSWK baculovirus recombinant. Lane 4 contains the proteins that were isolated from insect cells infected with the VP7-RVLAIERYLKD recombinant. Lysates from cells infected with the dual recombinant baculoviruses containing the VP7- RVLAIERYLKD-ALDSWK construct were run in lanes 5 and 6. Lane 7 contains the 39 KDa positive control band, the AHSV-9 VP7 mt 177 protein, expressed in insect cells by a VP7 baculovirus recombinant. Lanes 8 and 9 contain proteins isolated from wild type baculovirus infected and uninfected insect cells, respectively. No samples were run in lanes 1 and 10.

2.4 DISCUSSION

Various protein particulate strategies have been employed to display the HIV-1 B epitope ELDKWA to the immune system. These include the antigenic B loop site of the influenza virus (Muster *et al.*, 1994) and induced a humoral immune response at the mucosal level (Muster *et al.*, 1995; Ferko *et al.*, 1998). The sequence LLELDKWASL was also inserted into the V1, V2, V3 and V4 variable loops of gp120, generating HIV-1 IIIB Env mutants (Liang *et al.*, 1999). These results revealed that the precise location into which an epitope is inserted has a direct effect on the ELDKWA epitope's ability to induce neutralising antibodies. This suggests that the configuration of the epitope plays a critical role in eliciting a specific humoral immune response. Other presentation strategies include the hepatitis B virus (HBV) surface antigen (HbsAg) (Eckhart *et al.*, 1996), the loop region of the immunoglobulin heavy chain FR3 (Cook & Barber, 1997) and the baculovirus system (Ernst *et al.*, 1998; Ernst *et al.*, 2000). The ELDKWA epitope has also been expressed in *E.coli,* however these results confirmed that the system in which protein is expressed determines its ability to induce neutralising antibodies (Brown *et al.*, 1995; Coeffier *et al.*, 2000). However, none, with the exception of the novel 'epitope' vaccine strategy (Chen *et al.*, (1999a), were able to generate a strong ELDKWA-specific immune response.

A novel approach in presenting epitopes to the immune system is the utilisation of AHSV-9 VP7 crystalline structures. In order to determine whether this presentation system will be able to induce higher titres of antibodies than other particulate systems, the HIV-1 C sequences encoding RVLAIERYLKD and ALDSWK as well as their flanking regions were inserted into the top domain (position 177) of AHSV-9 VP7 gene. Precisely how this was accomplished follows below.

Due to some uncertainty over the precise nucleotide sequence of the multiple cloning site (MCS) inserted between nucleotides 548 and 549 of the native AHSV-9 VP7 gene (Maree, 2000), the donated pFASTBAC-VP7 mt 177 plasmid was sequenced. The sequence data confirmed the order of the restriction enzyme sites in the MCS to be 5' *Hind* III, *Xba* I and *Sal* I. Accordingly, the amino acids encoded by these additional nucleotide sequences, and present in the modified VP7 protein, were in the order N-terminal K, L, S, R, V, D. This data dissipated the previous suggestion by Maree, 2000

that the order of the restriction enzyme recognition sites in the MCS was 5' *Sal* I, *Xba* I and *Hind* III.

Also in question was the number of recognition sites for each of the candidate restriction enzymes to be utilised in subsequent cloning procedures. Restriction enzyme analysis of the pFASTBAC-VP7 mt 177 plasmid confirmed the presence of unique *Hind* III and *Sal* I recognition sites in the vector, as suggested by Maree, 2000. Also elucidated was the absence of *Eco* RI recognition sites and an indication that there were in fact two *Xba* I recognition sites present in the vector. The *Xba* I site has been expected to be unique and present in the inserted MCS sequence (Maree, personal communication). Sequencing data confirmed the presence of a second *Xba* I site in the region immediately adjacent to the 3' end of the cloned VP7 gene. Based on the above data, the *Xba* I restriction enzyme has two target sites in the pFASTBAC-VP7 mt 177 vector, and this indicated that only the *Hind* III, *Sal* I and *Eco* RI enzymes were suitable to be utilised in the cloning of inserts into the vector.

Nucleotide sequences representing the RVLAIERYLKD and ALDSWK amino acids as well as a number of adjacent nucleotides were amplified via PCR from the donated pFASTBAC-Du 151 gp 160 template DNA, utilising primers designed specifically for this purpose (Table 2.3). Inclusion of the nucleotides flanking the epitopes was an attempt to enable the RVLAIERYLKD and ALDSWK amino acids to attain their optimal configurations and induce high titres of neutralising antibodies.

The PCR products as well as the pFASTBAC-VP7 mt 177 vector were sequentially digested with restriction endonucleases *Hind* III and *Sal* I ligated and transformed into competent *E.coli* cells. The authenticity of the recombinant bacterial colonies was tested by means of the colony blot procedure and reconfirmed with the polymerase chain reaction (PCR). To determine whether the RVLAIERYLKD and ALDSWK-encoding nucleotides had been inserted into the vector without any frameshift mutations occurring, clones pFASTBAC-VP7- RVLAIERYLKD and pFASTBAC-VP7- ALDSWK were partially sequenced over the MCS. This was accomplished using the specially designed primer VP7FWD1, which binds to nucleotides 440-457 of the native AHSV-9 VP7 gene. Translation of the sequence data confirmed that no frameshift mutations had occurred during the cloning procedure.

After the cloning of the RVLAIERYLKD and ALDSWK-encoding nucleotides into the pFASTBAC-VP7 mt 177 vector individually, it was speculated that a stronger humoral immune response may be induced should both these regions be presented simultaneously to the immune system on the surface of the AHSV-9 VP7 protein (Huismans, personal communication). In order to test this theory and following a strategy similar to the one utilised to construct the single recombinant vectors, a dual recombinant vector, containing both the RVLAIERYLKD and ALDSWK nucleotide sequences, was constructed. After the digestion of the ALDSWK PCR product and the recombinant pFASTBAC-VP7 mt 177- RVLAIERYLKD vector with restriction enzymes *Sal* I and *Eco* RI, these fragments were ligated and transfected into *E. coli* cells. Restriction enzyme analyses were performed to identify possible recombinant bacterial clones. These results were confirmed via PCR. To determine whether any frameshift mutations had occurred during the insertion of the ALDSWK sequence into the vector, the pFASTBAC-VP7- RVLAIERYLKD-ALDSWK plasmid was partially sequenced over the MCS again utilising primer VP7FWD1. Sequencing analysis indicated that no frameshift mutations had occurred during the insertion of the ALDSWK sequence.

As previously noted, the native VP7 protein is able to tolerate an insertion of 100 amino acids in its top domain without the loss of its trimeric structure and ability to form crystals (Maree, 2000). It was to this end that the sequences amplified and inserted into the VP7 top domain did not greatly exceed the tentative limit of 100 amino acids. The ALDSWK region amplified was 48 amino acids in size whereas the amplified RVLAIERYLKD region was 51 amino acids. The insert containing both the above-mentioned sequences encoded 99 amino acids. Although the PCR primers were originally designed to amplify sequences encoding approximately 50 amino acids, the addition of restriction enzyme recognition sites onto the ends of these primers, to facilitate cloning, resulted in an additional six amino acids being added to the final chimeric VP7 proteins. Thus the ALDSWK region inserted intro position 177 of the native VP7 protein was a total of 54 amino acids in size, the inserted RVLAIERYLKD region was 57 amino acids and the RVLAIERYLKD-ALDSWK insertion in VP7 was 105 amino acids.

Their suitability determined by sequencing analyses, the pFASTBAC-VP7-RVLAIERYLKD, pFASTBAC-VP7- ALDSWK and pFASTBAC-VP7- RVLAIERYLKD-ALDSWK constructs were utilised in the construction of recombinant baculoviruses. To

determine whether the recombinant baculoviruses were indeed expressing the chimeric VP7 proteins, infected Sf9 cell monolayers were harvested and their proteins analysed by SDS-PAGE and Coomassie blue staining. Expression of the chimeric proteins VP7-ALDSWK, VP7-RVLAIERYLKD and VP7-RVLAIERYLKD-ALDSWK was detected in the infected cells.

By utilising the general assumption that six amino acids have a molecular weight of approximately one KDa (Maree, 2001), the molecular weights of the chimeric VP7 proteins were calculated. The molecular weight of protein VP7-ALDSWK was approximated to be 47.3 KDa while that of VP7- RVLAIERYLKD was found to be approximately 47.8 KDa. The VP7 protein containing both the RVLAIERYLKD and ALDSWK regions was calculated to be 55.8 KDa. However, the use of the above assumption in calculating only approximate values of the molecular weights of proteins became apparent when a inconsistency in the PAGE electrophoresis results was discovered. The chimeric VP7-ALDSWK protein band, which had a calculated molecular weight of 47.3 KDa, was in fact larger in size than the 47.8 KDa VP7- RVLAIERYLKD protein band. This inconsistency can however be explained by the fact that different amino acids contain different molecular groups, resulting in amino acids having varying molecular weights. The specific amino acids inserted with the ALDSWK epitope into the VP7 protein may therefore have a greater combined molecular weight than the amino acids inserted into VP7 together with the RVLAIERYLKD epitope.

Further research would involve the expression of large quantities of the VP7- ALDSWK, VP7-RVLAIERYLKD, VP7-RVLAIERYLKD-ALDSWK chimeric proteins in insect cells and their subsequent isolation and purification. The availability of these proteins will enable their characterisation and facilitate solubility and scanning electron microscopy studies. Injecting these proteins into small animals, followed by Elisa or Western blot procedures, will confirm whether the subtype C sequences ALDSWK and RVLAIERYLKD are able to induce an immune response when presented with their flanking amino acids on VP7 crystalline particles.

Although the immunogenic status of the HIV-1 C sequences ALDSWK and RVLAIERYLKD is yet to be established, the detection of a epitope-specific neutralising immune response would indicate the suitability of VP7 as a particulate presentation

system and confirm the epitope-status of ALDSWK and RVLAIERYLKD. A control chimeric protein, consisting of VP7 into which the HIV-1 B epitope ELDKWA has been inserted into position 177, is currently in the process of being synthesised (Fick, personal communication). Should no epitope-specific immune response be detected following the immunisation of small animals with the chimeric VP7 proteins, this control chimeric protein will be utilised in order to determine whether the negative result is as a consequence of the subtype C sequences not being immunogenic or whether AHSV-9 VP7 particulate system is not suitable as a epitope presentation system.

CHAPTER 3

CONSTRUCTION AND ANALYSIS OF A RECOMBINANT LSDV CONTAINING THE AHSV-9 VP7 GENE

3.1 INTRODUCTION

As already discussed, one of the long-term aims of this study is the development of AHSV-9 VP7 crystalline particles for the presentation of immunologically important epitopes to the immune system. However, should the chimeric VP7 construct contain CTL or T_H (T-helper) epitopes, delivery into the cellular environment is necessary in order to generate the appropriate cellular immune response. The development of the Lumpy Skin Disease virus (LSDV) as such a live recombinant viral delivery system is another of the long-term goals of this study.

In order to stimulate a strong, appropriate immune response against the foreign antigen that is delivered by the viral vector, the strength of the promoter directing the genes expression, as well as the stage during the infection cycle at which the promoter is activated, is important (cited by Fick, 1998).

Poxviral gene expression can be divided into two phases with early transcripts being synthesised prior to DNA replication, and late mRNAs synthesised after the onset of DNA replication. Early transcripts, encoding primarily for functional proteins, have been found less abundant than late transcripts encoding for structural proteins (reviewed in Moss, 1996). Thus 'late' promoters drive gene expression more strongly than early promoters, making late or early/late promoters attractive for controlling the expression of transgenes (Mastrangelo *et al.*, 2000). However, some studies indicate that the induction of a good cytopathic T-lymphocyte (CTL) response to foreign antigen requires early expression of that foreign gene product (Coupar *et al.*, 1986, Townsend *et al.*, 1988). In addition, previous studies indicate that homologous virus-promoter recombinants yield higher levels of marker gene expression than heterologous virus-promoter recombinants (Kumar & Boyle, 1990; Boyle, 1992). This could be because promoter recognition may not be uniform in different systems. This led the authors Fick & Viljoen, 1999 to identify and characterise an authentic LSDV bi-directional promoter subsequently to be utilised for the expression of foreign genes in a LSDV vector system.

The 56 bp pA7LA8R bi-directional promoter element is described as having both early (pA8R) and late (pA7L) transcriptional activities with transcription of the early viral genes in a rightward direction and the transcription of the late genes in a leftward direction (Fig 3.1). The bi-directional characteristic of this promoter is advantageous in that a foreign gene may be placed under the control of both the early and late transcriptional elements. Thus the gene may be expressed during the entire viral replicative cycle. Expression from the early promoter element would allow for the induction of a good CTL response, while a high yield of expression would be obtained from the late promoter.

Promising preliminary expression results from the early (pA8R) promoter element of the LSDV bi-directional promoter have already been obtained (Vos, 2001). The level of VP7 expression and crystal formation in fetal bovine testis (FBT) cells infected with an LSDV-VP7 recombinant was found to be higher than in comparable systems. It would therefore also be advantageous to determine whether the level of VP7 expression from the late (pA7R) promoter is greater than the expression level obtained from the early element.

In the first part of this study, the native AHSV-9 VP7 gene is to be cloned into the donated pHSsgpt transfer vector (Dr W. Fick, UP) under the control of the late LSDV promoter element pA7L (Fig 3.1). Also present in the pHSsgpt vector is the *E.coli* xanthine-guanine phosphoribosyl transferase (gpt) gene under the control of the vaccinia early/late p7.5K promoter. This marker gene serves as a means of dominant, positive selection as recombinant viruses containing this gene will exhibit resistance to the mycophenolic acid selective medium (Mulligan & Berg, 1981). Flanking the pA7LA8R promoter and the gpt selection marker are two LSDV genomic sequences, orthologues of the vaccinia virus A2L and A3L genes. The recent sequencing of the LSDV viral genome (Tulman *et al.*, 2001) has enabled the renaming of the LSDV A2L and A3L genes to LSDV092 and LSDV094, respectively. In addition the assignment of putative functions to these genes has also been made possible. The LSDV092 gene (84% homology to the vaccinia A2L gene) encodes a late transcription factor while LSDV094 (64% homology to the vaccinia A3L gene) encodes a virion core protein. For the purpose of this study, however, the LSDV sequences will be referred to as A2L and A3L. The sequences on the pHSsgpt vector dictate an intergenic site between A3L and A2L genes, into which the foreign gene is to be inserted into the LSDV genome. By definition the foreign gene, which should also contain its own translation start and stop signals to avoid improper reading

frames, has to be inserted into a region of the poxviral genome not essential for viral replication (Baxby, 1993). Following homologous recombination between the A2L and A3L sequences on the transfer plasmid and those on the wild type (wt) genome, the foreign gene should be integrated into the LSDV genome between the two genes that are analogous to vaccinia virus A2L and A3L genes (Fick, personal communication).

The second part of this investigation involves the generation of the recombinant LSDV-VP7 by utilising the process of homologous recombination (Mackett *et al.*, 1985 as cited by Fick, 1998). Integration of the AHSV-VP7 gene into the LSDV genome will be achieved by infecting the host lamb testis (LT) cells with wt LSDV and subsequently transfecting the cells with recombinant pHSsgpt-VP7 transfer vector. Homologous recombination between the A2L and A3L gene analogous flanking the VP7 gene and the LSDV genome (discussed above) will result in the production recombinant LSDV virus. Recombinants shall be subsequently selected for by making use of their resistance to mycophenolic acid. Expression of the VP7 gene from the late pA7L promoter of the recombinant virus will be determined by using the technique of SDS-PAGE and the sensitive Western blot procedure.

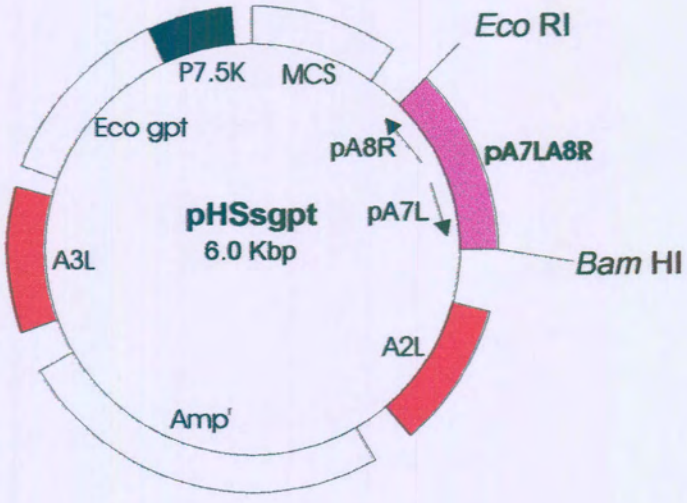

Fig 3.1 Diagramatic representaion of the pHSsgpt transfer vector plasmid. The unique bi-directional pA7LA8R promoter allows for the expression of foreign genes in both the early (pA8R) and late (pA7L) stages of the viral replication cycle (Vos, 2001).

3.2 MATERIALS AND METHODS

3.2.1 Restriction enzyme digestion

In order to obtain the gene of interest, double digestions were performed in a total volume of 50 µl, containing 14.52 µg of DNA and 30 Units (U) of each enzyme (Boehringer Mannheim). The transfer vector was linearised in a single digestion reaction of 20 µl, containing 5.61 µg of DNA and 10 U of restriction enzyme (Boehringer Mannheim). Each digestion was performed for 1,5 hr. Reaction conditions were as described in section 2.2.7. The digested DNA products were analysed by agarose gel electrophoresis on 1 % agarose gels.

3.2.2 Purification of DNA fragments excised from agarose gels

DNA fragments of interest were excised from a 1% agarose gel (Sambrook *et al.*, 1989) and purified by means of the Glassmilk method. This was done by making use of the Geneclean™ II kit (Bio101), according to the manufacturers instructions. Glassmilk is a specially formulated suspension of silica matrix that binds single or double stranded DNA without binding any contaminants. Two and a half volumes of saturated sodium iodide (NaI) solution was added to the excised agarose gel fragment. The addition of NaI ensures the presence of a high salt concentration thereby enhancing the binding of DNA to the silica matrix. The agarose was melted by placing the reaction at 55° C for 5 min, after which 5 µl of a Glassmilk™ suspension was added per 5 µg or less of DNA. An additional 1 µl of glassmilk suspension is added for each 0.5 µg of DNA above 5 µg. After placing the reaction on ice for 15 min, the silica matrix with bound DNA was pelleted by centrifugation at 12000 rpm for 1 min and washed three times with ice-cold NEW WASH (NaCl, Tris, EDTA, ethanol and water) (Bio101). NEW WASH, with an optimal salt concentration, ensures the DNA remains bound to the silica matrix while all contaminants are washed away. The DNA was eluted from the silica matrix by resuspending it in 10 µl of double distilled water (ddH_2O) and incubation at 55°C for 3 min. After centrifugation at 12000 rpm for 1 min and harvesting of the DNA-containing supernatant, the elution step was repeated to yield 0.6% of the original quantity of DNA in a final volume of 20 µl. One tenth of the recovered DNA was analysed by 1% agarose gel electrophoresis.

3.2.3 Generation of blunt ended DNA fragments

The Klenow reaction was employed to generate blunt-ended DNA molecules for cloning purposes. The Klenow enzyme acts by filling in the exposed 5' sticky ends of digested DNA molecules with complementary nucleotides. Three microgram of DNA was placed in a 25 µl reaction containing 5 mM dNTP's, 4 U of Klenow enzyme (Boehringer Mannheim) and a final 1× concentration of the appropriate buffer (Boehringer Mannheim).The reaction was incubated at 30°C for 30 min and then subjected to 65°C for 5 min to inactivate the Klenow enzyme. The desired DNA fragment was purified by means of the High Pure purification kit (Roche).

3.2.4 Dephosphorylation of vector DNA

In order to reduce the possibility that the blunt-ended vector DNA might religate during the ligation reactions, the calf intestinal Alkaline Phosphatase (AP) enzyme (Roche) was utilised. The enzyme removes 5' terminal phosphate residues on the ends of the vector DNA and prevents its recirculisation. 1U of AP, dephosphorylation buffer (0.5 M Tris-HCl, 1 mM EDTA, pH 8.5) to a final concentration of 1 × and water were added directly to the vector DNA, resulting in a final volume of 40 µl. The reaction was incubated at 37°C for 30 min, after which it was subjected to 65°C for 5 min to inactivate the enzyme. The contents of the reaction were analysed by 1% agarose gel electrophoresis and purified using the High Pure PCR purification kit (Roche).

3.2.5 Ligation of the vector and insert

Blunt-ended ligation of the insert DNA to the linearised, dephosphorylated vector was accomplished as described in section 2.2.8, with the exception that reactions were incubated at 22°C overnight. A control ligation reaction containing only linearised, dephosphorylated vector was prepared to test the efficiency of the dephosphorylation procedure.

3.2.6 Transformation

Half of the volume of the ligation reactions, the insert-vector ligation reaction as well as the control ligation reaction, were each added to 100 µl of XL-1 Blue competent cells on ice. The transformation procedure was performed as described in section 2.2.2, except that one third of each of the three transformation mixtures was plated out. As in 2.2.2, tetracycline and ampicillin resistant colonies were selected for screening.

3.2.7 Plasmid DNA isolation

Plasmid DNA was isolated from several Tet and Amp resistant bacterial clones using the small-scale plasmid purification procedure, described in section 2.2.3. When a colony harbouring the desired recombinant plasmid was identified by restriction enzyme digestion, a large-scale purification procedure was undertaken using the Nucleobond AX kit (Macherey-Nagel).

3.2.8 Passaging tissue culture cells

Primary Lamb Testis (LT) cells were kindly provided by Wilma Fick (UP) and David Wallace (OVI, Onderstepoort). All cell culture handling techniques were essentially as described by Summers & Smith, 1987. A cryopreservation tube (Simport), containing LT cells, was removed from storage at -70°C in liquid nitrogen and thawed at room temperature. The entire contents of the tube, approximately 1.8 ml, was seeded in 25 cm^2 culture flask (Nunc™) containing 10 ml of a 37°C-prewarmed 20% foetal calf serum cell culture growth medium (40% Dulbecco's Modified Eagles Medium (DMEM), 40% HAMS F12, 20% foetal calf serum (FCS) with antibiotics Penicillin G (120 µg/ml), Streptomycin Sulphate (120 µg/ml) and antimycotic Fungizone (0.3 µg/ml). All the components of the growth medium were manufactured by Highveld Biological. The flask was gently swirled to ensure an even spread of cells and placed in a 5% CO_2 incubator at 37°C for 4-5 hours. Following adhesion of the cells to the bottom of the flask, the medium was aspirated and replaced by 10 ml of fresh 20% foetal calf serum growth medium. The flask was left overnight in a 5% CO_2 incubator at 37°C. The following day, the adhered cells were examined under a microscope for cell growth.

Once the cells had formed a confluent monolayer they were passaged using the following protocol. The 20% foetal calf serum growth medium was aspirated off the cells and the confluent cell monolayer washed with 10 ml of 37°C-prewarmed 1 × Phosphate Buffered Saline (PBS-A) solution (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_20$, 0.14 mM KH_2PO_4. pH 7.3) for 2 min. Following removal of the 1× PBS-A, the cells were washed with 5 ml of 37°C-prewarmed 0.25% Trypsin/ 0.1% Versene (in Ca^{2+} and Mg^{2+} free phosphate buffered saline (Highveld Biological)), by swirling the trypsin over the cells. Almost all, save 500 µl, of the trypsin was removed and the flask placed in a 5% CO_2 incubator at 37°C. As soon as the cells detached from the bottom surface of the flask a small volume (2-3 ml) of 10% foetal calf serum growth medium (45% DMEM, 45% HAMS F12, 10% FCS with 120 µg/ml Penicillin G, 120 µg/ml streptomycin sulphate, 0.3 µg/ml fungizone), prewarmed to 37°C, was added. The cells were gently pipetted up and down using a 10 ml syringe (Promex) with an 18 gauge (G) needle (Promex) until no clumps were visible. Fifteen ml of prewarmed 10% FCS growth medium was added to a sterile 75 cm^3 culture flask (Nunc™). The entire cell suspension from the 25 cm^3 flask was seeded in the larger flask. This causes the cells to be divided in a split ratio of 1:3. Three to four days post sub-culturing, the number of confluent cells will be three times the number of cells present in the original parent monolayer. After gentle swirling to ensure an even spread of cells, the flask was placed in a 5 % CO_2 incubator

at 37°C until microscopy revealed a confluent cell monolayer. Approximately 4 days after sub-culturing the cells were once again divided in a split ratio of 1:3 and confluent cell monolayers were obtained in three 75 cm^3 culture flasks after three days. One of these monolayers was used for transfections.

3.2.9 Transfection procedures

A 6-well Nunc™ plate was seeded with the LT cell suspension of one of the 75 cm^3 flasks described above and left overnight at 37°C in a 5% CO_2 incubator. The following day, when the monolayers of cells were 90% confluent (approximately 3 × 10^5 cells/well), they were washed with DMEM supplemented with antibiotics (120 µg/ml Penicillin G, 120 µg/ml streptomycin sulphate, 0.3 µg/ml fungizone) and covered with 1 ml diluent (0.01 M Hepes (Sigma) in growth medium not containing FCS). The cells were infected with purified Lumpy skin disease virus stock (Neethling strain) at a multiplicity of infection (m.o.i) of 0.1 plaque forming units (pfu) per cell. Transfection of the recombinant transfer vector was performed 1 hr post-infection by making use of the Lipofectamine-mediated (Gibco-Brl, Life Technologies) transfer method as follows: 5 µl of lipofectamine and 95 µl of DMEM were gently mixed. A second mixture of 5 µg vector plasmid DNA made up to a total of 100 µl with DMEM was also prepared. The two mixtures were added together, gently mixed and left at room temperature for 1 hr. One hour post-infection the infected LT cells were washed with DMEM. Eight hundred µl of DMEM was added to the 200 µl plasmid-lipofectamine mixture. The total volume was added drop wise onto the cells. After a 7 hr incubation period in the 5% CO_2 incubator at 37°C, 1 ml of 10% FCS growth medium (without antibiotics and antimycotics) was added and the medium swirled gently but thoroughly. The cells were left overnight in the 5% CO_2 incubator at 37°C and 24 hours post-infection the supernatant was removed from the cells. Two ml 10% FCS growth medium with antibiotics (120 µg/ml Penicillin G, 120 µg/ml streptomycin sulphate, 0.3 µg/ml fungizone) was added and the cells were incubated in the 5% CO_2 incubator at 37°C.

3.2.10 Selection for LSDV recombinant virus

Six days post-infection (p.i), when a good cytopathic effect (CPE) was visible on the LT cells, the supernatant was harvested and subjected to three freeze-thaw cycles. The virus-containing supernatant was stored at -20°C to be used for future infections of LT cells. Monolayers of 90% confluent LT cells in a 6-well plate were each incubated in 3 ml of mycophenolic acid (MPA) selection medium 24 hrs prior to infection. The procedure of incubating the LT cells in selective medium one day prior to infection, was repeated for all subsequent infections. The MPA selection medium is composed of 30 µg/ml mycophenolic acid (Sigma), 250 µg/ml Xanthine (Sigma), 200 µg/ml HAT (13.6 µg/ml hypoxanthine, 0.16 µg/ml aminopterin and 3.84 µg/ml thymidin) from Highveld biological, and 2.5% FCS in DMEM supplemented with 120 µg/ml Penicillin G, 120 µg/ml streptomycin sulphate, 0.3 µg/ml fungizone. Just before infection, the transfection supernatant was thawed at 37°C for 3 min, placed on ice and sonicated for 30 sec in a Ultrasonic cleaner (Branson) at 4°C. The medium was removed from one of the 35 mm wells and 1 ml of the virus-containing supernatant was slowly pipetted into the well. Virus was adsorbed for 1,5 hr at 37°C in the 5% CO_2 incubator. 1 × PBS-A was used to wash the infected cells and was replaced with 2 ml of fresh MPA selection medium. During the selection period, the MPA selection medium had to be replaced every 72 hr with fresh medium in order to keep conditions consistent.

At 4 days p.i, the supernatant of the infection was harvested and used in a dilution series to infect new LT cell monolayers in a second round of selection. Supernatant was harvested off those cells that exhibited quite a number of plaques (100) but not complete CPE after incubation for 6 days. Following this third round of selection, the selection medium was replaced with 2 ml of 1 × PBS-A to allow for easier visualisation of plaques. Several single plaques were picked and inoculated into eppendorf tubes containing sterile selection medium. The selected plaques were subjected to another round of purification by making a short dilution series of each plaque in the presence of selection medium. When plaques became

visible, supernatant and cells from each of the wells of the 24 well plate (Nunc™) were harvested. The cells were harvested using trypsin/versene, centrifuged at 1500 rpm for 5 min and resuspended in 50 µl selection medium. Virus was released from the LT cells by three consecutive freeze-thaw cycles, after which the viral DNA was isolated, as is described in 3.2.13. The supernatants, however, were used to infect confluent LT cells on a 24-well plate. The supernatants containing recombinant LSDV virus, positively identified by PCR (3.2.14), were used to infect further LT cell monolayers and a single recombinant clone was amplified in a large scale manner.

3.2.11 Preparation of a LSDV viral stock

The culture medium was removed from eight 75 cm^3 flasks containing infected cells that exhibited 90% C.P.E. After carefully rinsing the cell monolayer with 1 × PBS-A, the infected cells were dislodged from the bottom of the flask using 5 ml trypsin/versene, prewarmed to 37°C. The cells were pooled with the medium and centrifuged at 1500 rpm for 15 min at 4°C using a Du Pont HS-4 rotor. The virus-containing supernatant was transferred to a sterile centrifuge tube. The cell pellet was resuspended in 2 ml McIlvains buffer (0.18 mM Citric acid, 3.63 mM $Na_2HP0_4.12H_20$ pH 7.4) and placed on ice for 10 min. The cell suspension was daunced ten times with a 7 ml daunce tissue grinder (Wheaton) and the cellular debris removed by centrifugation for 8 min at 2000 rpm. The supernatant was collected while the cell pellet was again resuspended in 2 ml McIlvains buffer to be incubated, daunced and centrifuged under the same conditions. The supernatant was collected and pooled with the one previously collected in a sterile centrifuge tube. Both supernatants were centrifuged at 13000 rpm for 1 hr at 4°C through a cushion of 36% sucrose in 1 × TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0) using a Beckman JA-25.50 rotor. Pellets containing virus were resuspended in a total volume of 300 µl DMEM and the viral stock stored at -20°C.

3.2.12 Virus Titration

A 6-well Nunc™ plate was seeded with LT cells and incubated at 37°C in a 5% CO_2 incubator. When the cells had reached 90% confluence, the culture medium was removed and replaced with MPA selection medium, 24 hrs prior to infection. A 10-fold dilution series of the LSDV stock was prepared in selective medium and 5 wells were infected with the entire contents of each dilution. One well on the plate was left uninfected as a control. The infection procedure followed was as described in section 3.2.10. The plate was incubated at 37°C until distinct plaques were visible. By counting the number of plaques in a well, where between one and ten plaques were visualised, and taking the dilution factor into account, the concentration of the original stock virus was determined.

3.2.13 LSDV DNA isolation and purification

In order to test for the presence of recombinant virus, viral DNA was isolated and purified according to the protocol described for vaccinia viruses (Mackett *et al.*, 1985 as cited by Fick, 1998):

Recombinant virus was isolated from the infected cell monolayers of eight 75 cm^3 culture flasks as described above in 3.2.11. An equal volume of lysis buffer (1% SDS, 20 mM β-mercaptoethanol, 20 mM EDTA) and Proteinase K to a final concentration of 100 µg/ml were added and the viral mixture incubated at 37°C overnight. The viral DNA was purified from residual proteins by a phenol-chloroform-isoamylalcohol (25:24:1) extraction. The aqueous phase was transferred to a new tube and extracted with an equal volume of chloroform-isoamylalcohol (24:1). Viral DNA was precipitated by the addition of one tenth of the volume of 3 M NaOAc pH 4.8 and 2.5 volumes 96% ethanol. After incubation at -20°C for 1 hr, the DNA was collected by centrifugation at 13000 rpm for 20 min at 4°C. Following a thorough 70% ethanol wash step, the DNA was pelleted again by centrifugation under the same conditions. All excess 70% ethanol was removed and the viral DNA was air-dried in a flow

laminar cabinet for approximately 30 min. Following the addition of 50 µl of ddH_20, the DNA was left to dissolve and was stored at 4°C.

3.2.14 Identification of recombinant clones via PCR

The Polymerase Chain Reaction (PCR) was utilised to identify recombinant LSDV clones that contained the desired gene in their viral genome. Viral DNA templates were added into PCR reactions containing various combinations of oligonucleotide primers. These included primer pair SON2a and SON2b, specific for AHSV-9 VP7, primer pair OP48 and OP49, specific for the LSDV Thymidine Kinase (TK) gene, and primers Harry and Sally, specific for the LSDV A2L and LSDV A3L genes, respectively (Table 3.1). Each set of primers was expected to yield specific amplification products after PCR (Table 3.2). The 50 µl PCR reactions contained approximately 50 ng viral genomic DNA and identical quantities of all the PCR components mentioned in 2.2.6. PCR reactions containing approximately 50 ng of wild type LSDV template DNA were also prepared, each one containing a set of primers mentioned above. Two positive control reactions, each containing 50 ng of DNA, were prepared as was a negative control reaction containing no template DNA. The reactions were performed in a Perkin Elmer GeneAmp PCR system 9600 machine. The reaction conditions were as follows: the DNA template was denatured at 95°C for 5 min. This step was followed by 30 cycles of denaturation (95°C for 1 min), primer annealing (45 sec at a temperature approximately 5°C below the Tm of the primer set (Table 3.1) and elongation (72°C for 2 min). A final elongation step of 4 min at 72°C was also performed. The PCR products were analysed electrophoretically on a 1% agarose gel with DNA molecular weight markers.

Table 3.1 List of oligonucleotide primers*

Primer	Sequence	Tm $4(G+C)_n + 2(A+T)_n$	Target
SON2a	5'd(CACAGATCTTT CGGTTAGGATGGA CGC-G)	66.8°C	AHSV-9 VP7 (8-26bp)
SON2b	5'd(CACAGATCTGT AAGTGTATTCGGTA TTGA-C)	56.9°C	AHSV-9 VP7 (1147-1167bp)
OP48	5'd(CCATGTATCTG CCATATCAAC)	60°C	LSDV TK (688-708bp)
OP49	5'd(GTGCTATCTSG TCGAGCTAT)	58°C	LSDV TK (835-854bp)
Harry-WF	5'd(GCATCAACATC TTCAGAAG)	52.4°C	LSDV A2L-specific
Sally-WF	5'd(GATAAACCACA GAAATGC)	49.1°C	LSDV A3L-specific

* Kindly reprinted, with permission, from the Masters thesis of Nadine Vos

Table 3.2 Sets of primers utilised and sizes of the expected PCR amplification products*

Primer set	DNA template	Annealing temp	PCR product (bp)
SON2a and SON2b	AHSV-7/9 VP7	56°C	1159
OP48 and OP49	wt LSDV TK	48°C	166
Harry-WF and Sally-WF	wt LSDV (A2L and A3L)	45°C	474
Harry-WF and Sally-WF	Recombinant LSDV/VP7 (A2L and A3L)	45°C	2959

* Kindly reprinted, with permission, from the Masters thesis of Nadine Vos

3.2.15 Non-radioactive labelling of DNA probes

In preparation for the Southern blot procedure, specific DNA probes as well as the λ II and φX markers were labelled using the DIG DNA labelling and Detection kit (Boehringer Mannheim), as described in section 2.2.10. Purification and quantification of the Dig-labelled probes was also performed according to the protocol in section 2.2.10. The DIG-labelled probes were subsequently quantified according to the protocol described in the DIG manual.

3.2.16 Restriction enzyme digestion and electrophoresis of viral DNA

Recombinant LSDV DNA, the wild type LSDV DNA and genomic DNA of uninfected LT cells, were digested with restriction enzymes (Boehringer Mannheim). The 40 µl reaction mixture, with recombinant LSDV DNA, contained approximately 12 µg viral DNA and 1 U of enzyme per µg of DNA. The two control reactions, with the wild type LSDV DNA and DNA of the uninfected LT cells, each contained 10 µg of DNA and 10 U of enzyme in a total volume of 40 µl. The reactions were incubated at 37°C for 3 hr. The digestion products were analysed by 1% agarose gel electrophoresis, after which they were concentrated into a smaller volume using a vacuum centrifuge. The contents of the reactions were equally divided and loaded in replica in a 0.6% agarose gel. Also loaded twice was 1 µg of DNA molecular weight marker λII and 4 µg of marker φX. Electrophoresis was carried out at 44 Volts (V) and 31 mA for approximately 8 hours.

3.2.17 Southern blot analysis

In order to confirm the presence of the desired gene in the recombinant viral clone, a Southern blot analysis was performed. Restriction enzyme digested genomic DNA, separated on a 0.6% agarose gel by electrophoresis (3.2.16), was transferred to a Hybond N^+ nylon membrane (Amersham) using a procedure developed by Southern (1975). Following the protocol described by Sambrook *et al.*, (1989), the DNA present in the agarose gel was firstly denatured. The 0.6% agarose gel was exposed to UV radiation for 1 min in order to nick the DNA. The gel was soaked, twice for 10 min, in 400 ml depurination solution (0.25 M HCl) with gentle agitation. After rinsing the gel two times with ddH_2O, it was placed in 200 ml denaturation solution (1.5 M NaCl, 0.5 M NaOH), with gentle agitation, for 20 min. This step was repeated using fresh denaturation solution. The gel was again washed twice in distilled water and its high pH neutralised by soaking in neutralisation solution (1 M Tris-HCl pH 8.0, 1.5 M NaCl, 10 mM EDTA) for 30 min with gentle agitation. Neutralisation was repeated using fresh neutralisation solution.

The single-stranded DNA fragments present in the agarose gel were then transferred to the Hybond N^+ nylon membrane using Southern's capillary transfer method, as described by Sambrook *et al.*, (1989). Paper towels were soaked with 20 × SSC (0.3 M Na_3citrate.$2H_2O$, 1.5 M NaCl pH 7.0), the transfer buffer, and stacked at the bottom of a plastic container also containing 20 × SSC. Six pieces of Whatmann 3 MMchr filter paper were soaked in 20 × SSC and placed on the wet towels. Air bubbles were removed by rolling a glass pipette over the stack. The agarose gel was placed, face down, on the soaked filter paper pieces followed by the nylon membrane, pre-soaked in 2 × SSC (30 mM Na_3citrate.$2H_2O$, 150 mM NaCl pH 7.0). A further 6 pieces of filter paper, pre-soaked in 2 × SSC, were placed on the membrane and the air bubbles removed. Three dry filter papers and pre-cut paper towels were then placed on the stack, followed by a perspex plate and a 0.5 kg weight. The blotting tower was left overnight at room temperature to ensure successful DNA transfer. The following day, positions of the wells were marked on the membrane, the gel removed and membrane washed in 2 × SSC. After the membrane had air-dried, the transferred DNA was fixed onto it by exposure to UV-light, 5 min on each side. The membrane was sealed in a plastic bag and stored at 4°C until use.

Probe hybridisation and Immunological detection of hybridised probe

Treatment of the nylon membrane, prior to, during, and post hybridisation, as well as the immunological detection of hybridised probes, was according to the protocol in section 2.2.10.

3.2.18 Protein isolations

Ninety percent confluent LT cells in a 6-well plate (Nunc™) were infected with virus stock at a m.o.i of 0.1 pfu/cell, in the presence of selective medium, as described in section 3.2.10. At various time intervals post-infection, the culture medium was removed from the well and the infected cells harvested with 1 × PBS-A by passing the cell suspension several times through a pipette tip. The harvested cells were centrifuged at 1500 rpm for 5 min at room temperature, washed twice with 1 × PBS-A and resuspended in 20 µl NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris pH 8.0, 100 µg/ml PMSF) at 4°C. Proteins were isolated from wild type LSDV infected and uninfected LT cells and served as controls. The samples were stored at -20°C until use.

3.2.19 Protein electrophoresis and Coomassie blue staining

One third of the volume of each sample was loaded onto a polyacrlyamide gel and the proteins separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE), as described in section 2.2.18. The polyacrylamide gel was subsequently stained in Coomassie Brilliant Blue staining solution to visualise the proteins (2.2.18).

3.2.20 Western blot analysis

In order to confirm expression of the desired gene by the recombinant lumpy skin disease virus, a Western blot analysis was performed. A replica of the 12% SDS polyacrylamide gel described in 3.2.19, containing identical protein samples, was electrophorised under the same conditions. One piece of Hybond C extra nitrocellulose membrane (Amersham) and two pieces of Whatman 3 MMChr filter paper were cut to the size of the unstained polyacrylamide gel, and together with the gel, were soaked in 1 × Towbins transfer buffer (25 mM Tris, 192 mM Glycine pH 8.3) for 30 min. Two Scotch Brite™ Sponge pads were squeezed and soaked twice in the transfer buffer. A blotting sandwich was assembled in an EC 140 Mini electroblot module, starting on the frame stand with stainless steel grid cathode. The first Scotch brite™ sponge pad was placed on the grid cathode, followed by a sheet of soaked filter paper and the polyacrylamide gel. The soaked nitrocellulose membrane was placed directly on the gel, followed by another sheet of soaked filter paper. To ensure that no air-bubbles were trapped between the layers, a pipette was rolled over the stack after each step. After the second soaked Scotch brite™ sponge pad completed the assembly, the blotting stack was covered with the palladium wire anode, turned upright and clamps attached to the sides of the module. Transfer buffer was added until the compartment was full, the assembled module was inserted into the outer tank and the safety cover placed on top. The transfer process was performed at 15 V for 45 min and 24 V for another 45 min. Satisfactory transfer was confirmed by staining the gel with Coomassie Brilliant Blue staining solution as in 2.2.18.

After transfer, the membrane was rinsed for 5 min in 1 × PBS. Following the addition of blocking solution (1% (m/v) milk powder in 1 × PBS), the membrane was incubated at room temperature for 1 hr to block the non-specific binding sites. The blocked membrane was sealed in a plastic bag and incubated in a primary antibody solution, consisting of polyclonal anti-AHSV 9-VP7 guinea pig antibody diluted 1:100 in blocking solution. Incubation was performed overnight at room temperature with gentle agitation to allow binding of the antibodies to immobilised protein. The membrane was removed and washed three times with wash buffer (0.05% Tween in 1 × PBS), 5 min for each wash. A secondary antibody Peroxidase-conjugated Protein A (ICN), specific for the primary antibody and labelled with the enzyme horseradish peroxidase, was diluted in blocking solution, according to the supplier's instructions, and added to the membrane. After incubation at room temperature for 1 hr with agitation the membrane was washed three times in wash buffer, 5 min for each wash, and

once in 1 × PBS. The enzyme substrate solution was prepared as follows: A solution containing 60 mg 4-chloro-1-naphtol in 20 ml ice-cold methanol and a solution containing 60 µl hydrogen peroxide in 100 ml 1 × PBS were mixed together, just prior to use. The membrane was transferred to the enzyme substrate and incubated at room temperature until bands became visible. To stop the colour precipitation reaction, the membrane was rinsed with dH_2O.

3.3 RESULTS

3.3.1 Construction of the recombinant transfer vector pHSsgpt-VP7

The transfer vector plasmid pHSsgpt, depicted in Fig 3.1, was constructed by Dr W.C. Fick. This vector contains a bi-directional LSDV promoter, pA7LA8R. Unique *Eco* RI and *Bam* HI sites are located on either side of this promoter. Should a gene be cloned into the *Eco* RI site, it would be placed under the control of the early promoter, pA8R, whereas a gene cloned into the *Bam* HI site would be under the control of the late promoter, pA7L. The AHSV-9 VP7 gene was excised from the pBS-VP7 in T7 plasmid (pBS-T7) (available from UP Genetics Department) with restriction endonucleases *Sma* I and *Sal* I. The pHSsgpt vector was linearised with restriction enzyme *Bam* HI. Both the digested vector and VP7 insert DNA were subsequently blunt-ended by the Klenow enzyme. Following vector dephosphorylation the VP7 gene was cloned under the control of the LSDV late promoter, pA7L, creating the recombinant transfer vector pHSsgpt-VP7, schematically represented in Fig 3.2.

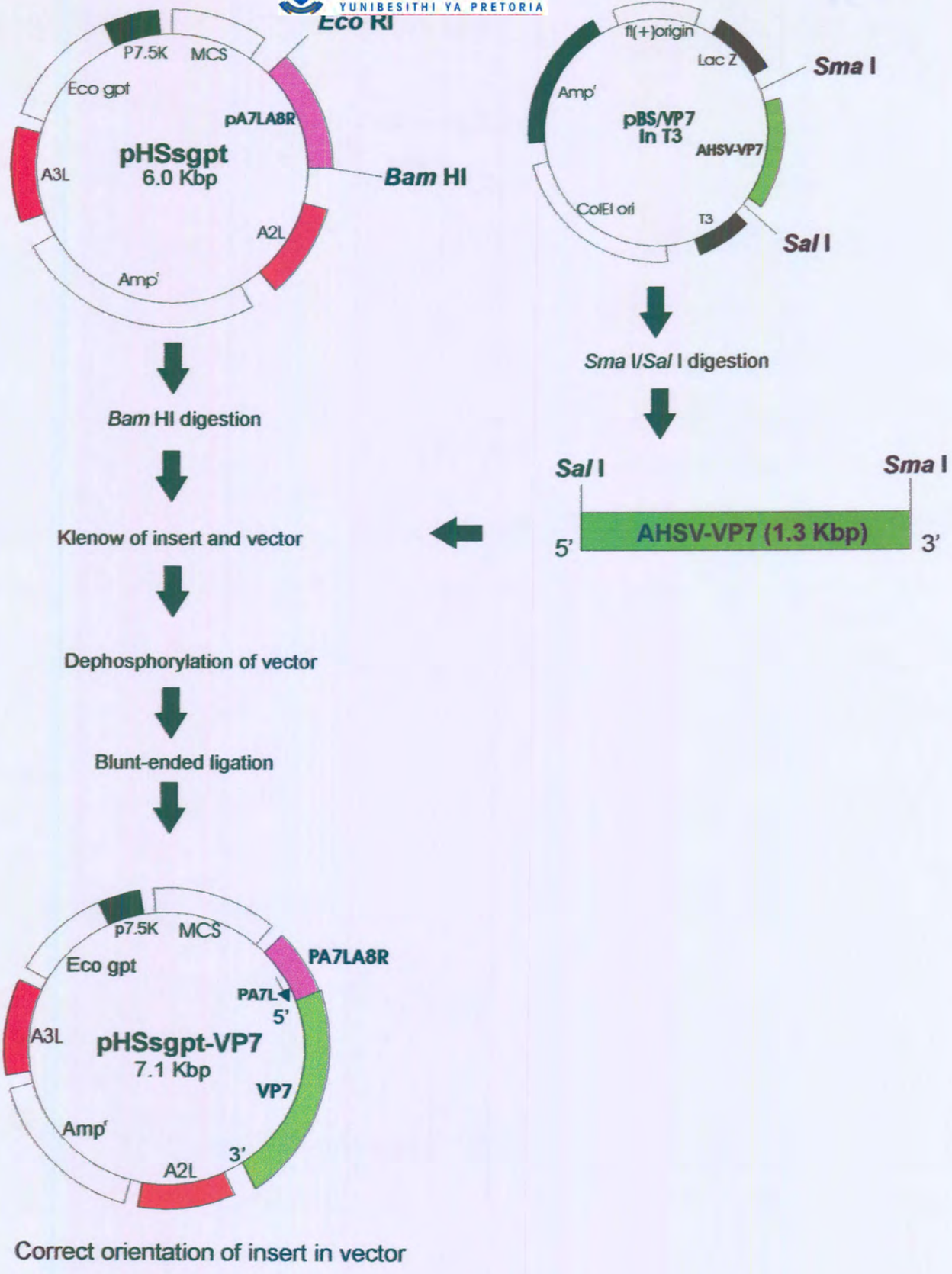

Fig 3.2 Schematic representation of the protocol followed to generate the recombinant transfer vector, pHSsgpt-VP7. The VP7 gene was cloned under the control of the late promoter, pA7L.

The transfer vector pHSsgpt and the donor plasmid pBS-T7 in T7 were kindly donated by N. Vos (UP). In order to obtain VP7, the gene of interest, plasmid pBS-T7 was sequentially digested with restriction endonucleases *Sma* I and *Sal* I (section 3.2.1). The transfer vector, pHSsgpt, was linearised with restriction enzyme *Bam* HI (3.2.1). Electrophoretic analysis of the digested products is shown in Fig 3.3. The 1.1 Kb insert band (Fig 3.3, lane 3) and the 6 Kb linearised vector band (Fig 3.3, lane 4) were excised from the 1% agarose gel and purified by the Glassmilk method (section 3.2.2). To determine the success of the purification procedure, a small quantity of the recovered DNA was analysed by 1% agarose gel electrophoresis (Fig 3.4). Blunt ended insert and vector DNA molecules were generated by means of the Klenow reaction to facilitate cloning (section 3.2.3). To reduce the possibility of vector religation, the pHSsgpt plasmid was dephosphorylated (section 3.2.4). Ligation reactions were prepared as described in section 2.2.8 and incubated overnight at 22°C, the optimal temperature for blunt-ended ligations. The ligation reactions were transformed into XL-1 Blue cells, as described in section 3.2.6. Ampicillin and tetracycline resistant colonies were randomly screened for the presence of recombinant plasmid by isolating plasmid DNA from several clones (section 3.2.7) and digestion with restriction enzymes *Sma* I and *Sal* I. The digestion products were analysed by 1% agarose gel electrophoresis (Fig 3.5). The presence of a DNA band (Fig 3.5, lane 6), identical in size to the 1.1 Kb VP7 gene band of the positive control (Fig 3.5, lane 3), indicated the presence of recombinant plasmid in lane 6 (Fig 3.5). In contrast, the plasmids in lanes 4,5 and 7,8 did not yield a 1.1 Kb digestion product and were concluded to be non-recombinant. The vector bands of all the digested plasmids, the recombinant included, were identical in size to the linearised 6 Kb pHSsgpt vector band (Fig 3.5, lane 9).

To determine the orientation of the insert, the recombinant plasmid DNA was digested with restriction enzyme *Sac* I in a single reaction, as well as a double digestion reaction with *Bam* HI and *Eco* RI. The digestion products were analysed electrophoretically on a 2% agarose gel (Fig 3.6). The 2 bands, 6.95 Kb and 150 bp in size, observed in lane 2 indicate that the VP7 gene was inserted in the correct orientation into the pHSsgpt vector (Fig 3.7a). Should the VP7 gene have been inserted in the incorrect orientation, digestion of the plasmid with restriction enzymes *Bam* HI and *Eco* RI would have yielded bands of 1.1 Kb and 6 Kb (Fig 3.7b). The presence of three *Sac* I digestion products (6.195 Kb, 750 bp, 155 bp) in lane 3 (Fig 3.6) confirms that the insertion was in the correct orientation. The recombinant

transfer vector, pHSsgpt-VP7, was subsequently used in the generation of an LSDV-VP7 recombinant.

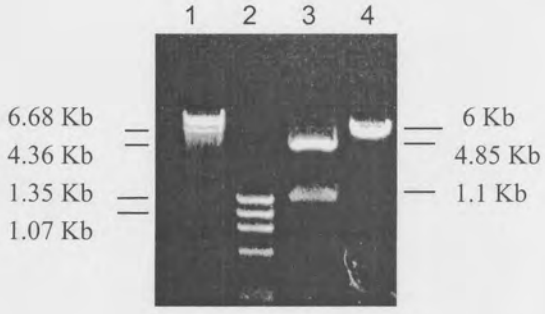

Fig 3.3 Agarose gel analysis of plasmids pBS-T7 and pHSsgpt, digested with restriction enzymes *Sma* I/*Sal* I and *Bam* HI, respectively. Lane 1 contains the DNA molecular weight marker λII, while lane 2 contains marker ϕX. The relevant sizes are indicated in Kb. Lane 3 contains the DNA fragments obtained following digestion of plasmid pBS-T7 with restriction enzymes *Sma* I and *Sal* I. Lane 4 contains the DNA fragments obtained after digestion of the pHSsgpt plasmid with restriction enzyme *Bam* HI.

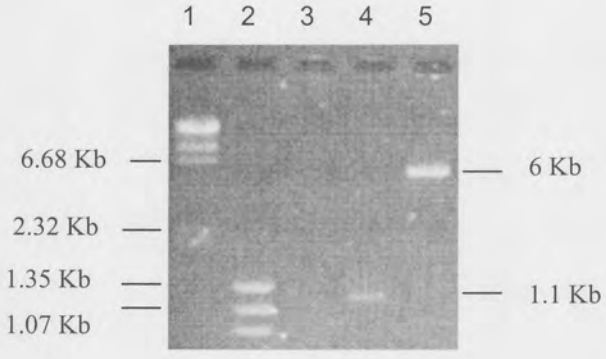

Fig 3.4 Agarose gel analysis of the VP7 insert and the linearised vector pHSsgpt following glassmilk purification and recovery of the DNA. Lanes 1 and 2 contain the DNA molecular weight markers λII and ϕX, respectively. The relevant sizes are indicated in Kb. The 1.1 Kb fragment containing the purified VP7 insert DNA is visualised in lane 4, while the linearised purified pHSsgpt vector band can be observed in lane 5. No sample was loaded in lane 3.

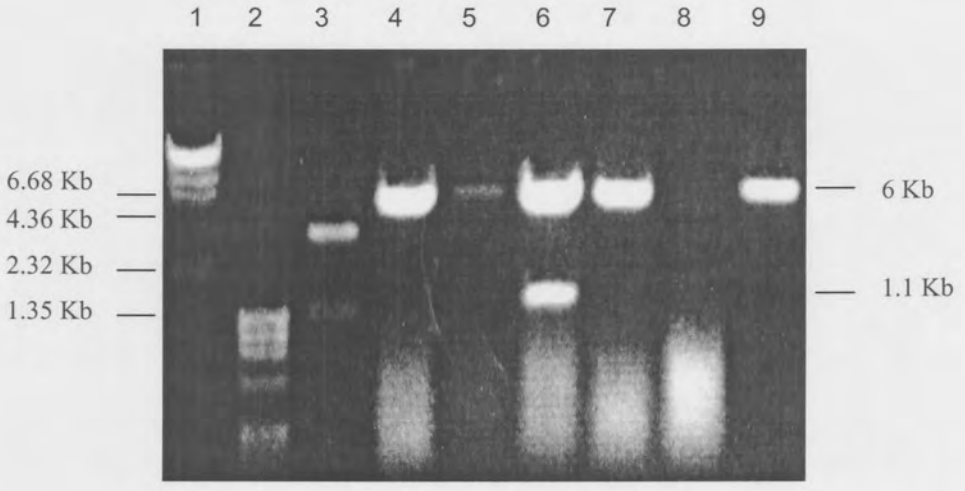

Fig 3.5 Agarose gel electrophoretic analysis of candidate recombinant plasmids after sequential digestion with restriction enzymes *Sma* I and *Sal* I. Lanes 1 and 2 contain the DNA molecular weight markers λII and ϕX, respectively. The relevant sizes are indicated in Kb. Following digestion of plasmid pBS-T7 with restriction enzymes *Sma* I and *Sal* I (positive control), two bands of 1.1 Kb and 3 Kb were visible (Lane 3). The fragment containing the VP7 gene is 1.1 Kb in size, while the remaining digested pBS vector is 3 Kb. A 1.1 Kb band can be visualised in lane 6, which contains the digested products of a recombinant clone. The linearised 6 Kb pHSsgpt vector band (lane 9) is also observed in each of the lanes 4-7. Lack of bands in lane 8 indicates degradation of plasmid DNA.

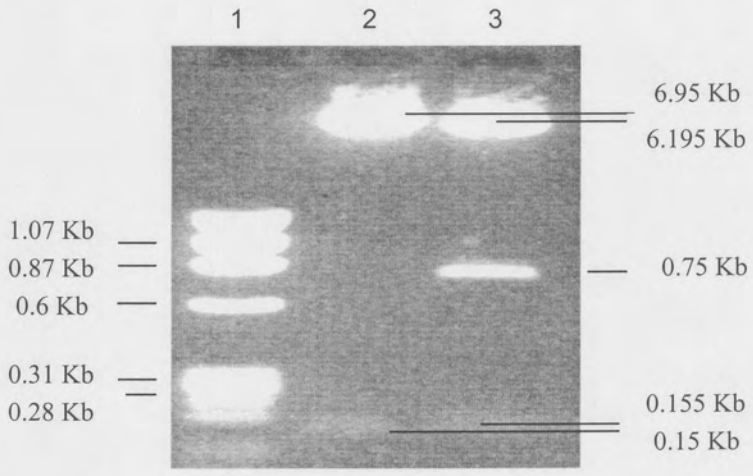

Fig 3.6 Two % agarose gel analysis of recombinant plasmid pHSsgpt-VP7, after digestion with restriction enzymes *Bam* HI/*Eco* RI and *Sac* I. Lane 1 contains DNA molecular marker ϕX. Relevant sizes are indicated in Kb. Two bands, 6.95 Kb and 0.15 Kb, can be observed in lane 2, following digestion with restriction enzymes *Bam* HI and *Eco* RI. Digestion with *Sac* I yielded three fragments 6.195 Kb, 0.75 Kb and 0.155 Kb in size (lane 3).

Correct orientation

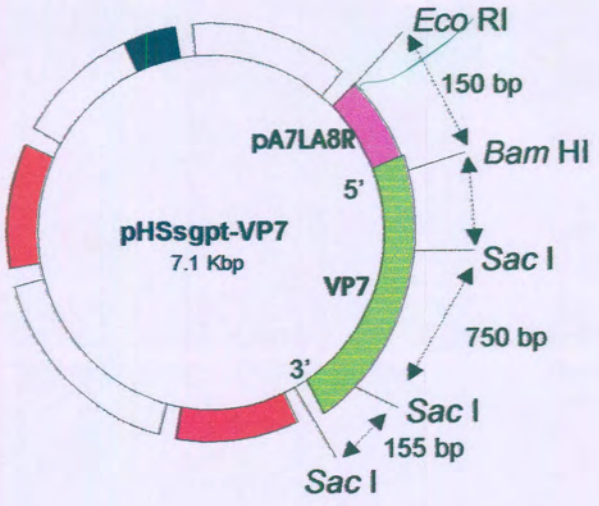

Fig 3.7a Diagrammatic representation of the recombinant pHSsgpt-VP7 plasmid, with the VP7 gene inserted in the correct orientation. A double digestion with *Eco* RI and *Bam* HI yields fragments 6.95 Kb and 150 bp in size, whereas *Sac* I digestion of the recombinant vector produces bands of 6.195 Kb, 750 bp and 155bp.

Incorrect orientation

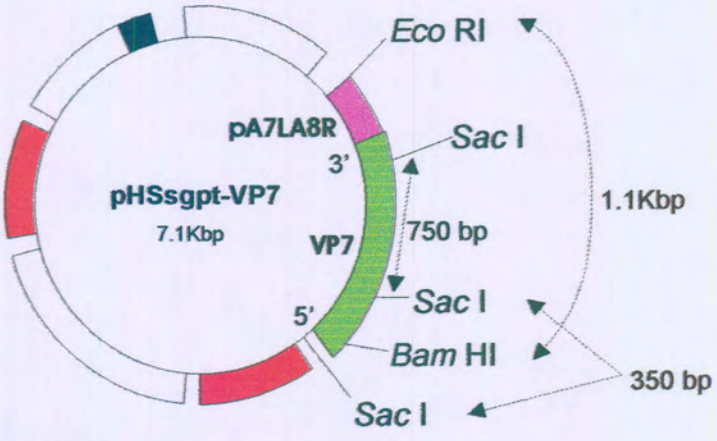

Fig 3.7b Diagrammatic representation of the recombinant pHSsgpt-VP7 plasmid, with the VP7 gene inserted in the wrong orientation. A double digestion with *Eco* RI and *Bam* HI yields bands of 1.1 Kb and 6 Kb, whereas digestion with *Sac* I yields fragments of 6 Kb, 750 bp and 350 bp.

3.3.2 Generation of a LSDV recombinant

Lamb testis (LT) cells were transfected with the pHSsgpt-VP7 transfer vector, as described in section 3.2.9. The process of homologous recombination and the products, resulting from the double cross over event between the wt LSDV DNA and pHSsgpt-VP7, are depicted in Fig 3.8. The supernatant, harvested from the transfected cells, was used to infect LT cells and the cells incubated in MPA medium to select against wt LSDV (section 3.2.10). Following three rounds of selection, single plaques were picked and each subjected to a short dilution series. Cells exhibiting CPE were harvested and viral DNA isolated, as described in section 3.2.13. Identification of recombinant plaques was performed by means of PCR.

3.3.3 Identification of recombinant clones via PCR

To determine which plaques contained the recombinant LSDV-VP7 virus, PCR reactions, utilising primer pairs Son 2a and Son 2b, were performed according to the protocol in section 3.2.14. The PCR products were analysed by 1% agarose gel electrophoresis (Fig 3.9). The primer pair, specific for the AHSV-9 VP7 gene, amplified a 1.1 Kb fragment from the viral DNA of several plaques (lanes 4-7,9). These bands were identical in size to the VP7 gene band amplified from the pBS-T7 positive control DNA (lane 11). Although the bands are less intense than the positive control band, it can be concluded that these plaques contain recombinant virus. The absence of the 1.1 Kb amplification product in the wt LSDV control (lane 10) indicates that the Son 2a and Son 2b primers were not able to bind to non-target sequences on the wt LSDV viral DNA. This result lends credibility to the argument that the 1.1 Kb bands in lanes 2 and 3 are indeed amplified from the VP7 DNA sequence. Also, the lack of bands in lane 12, containing the negative control, shows that no contamination with foreign DNA occurred during PCR preparation.

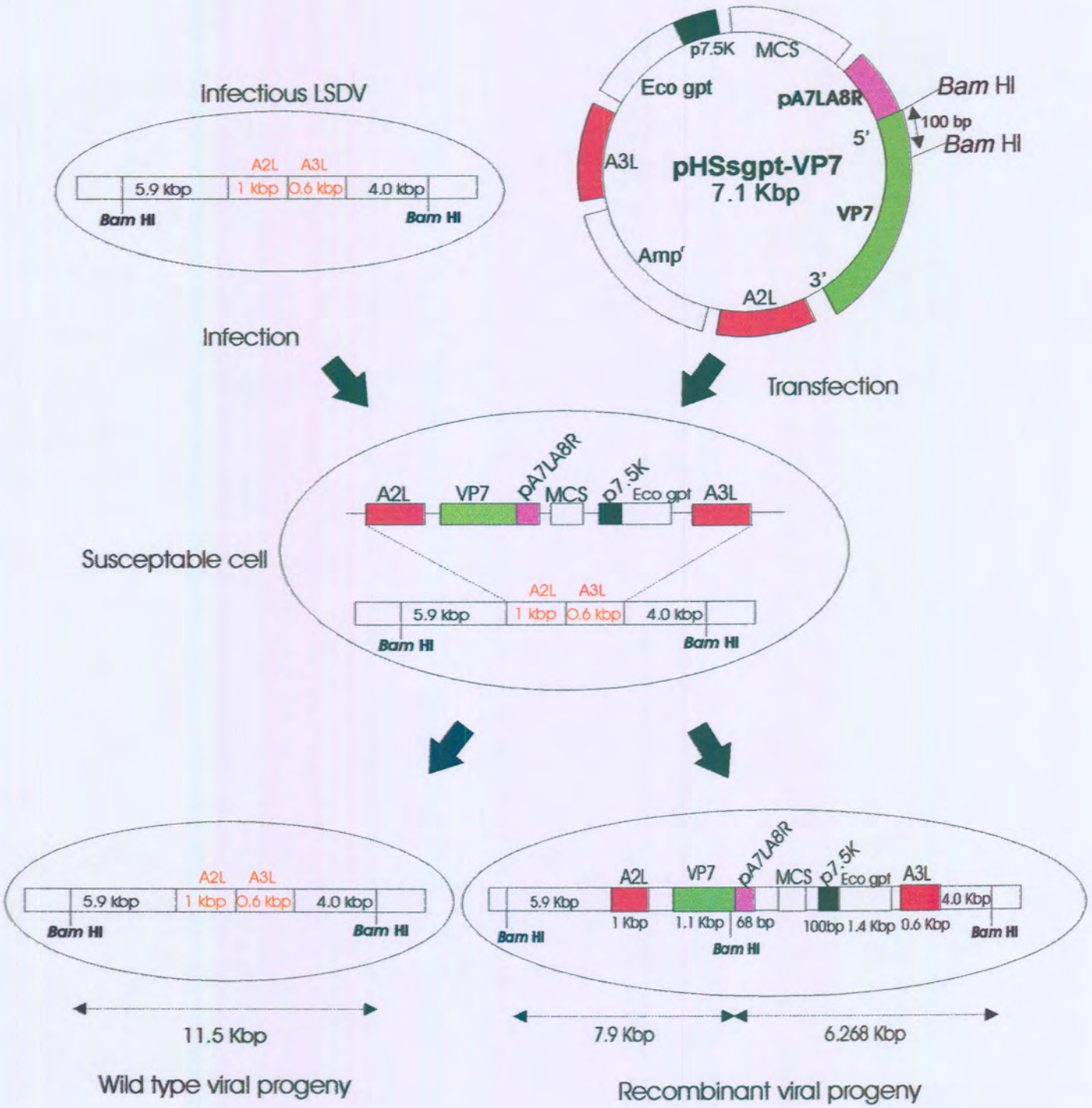

Fig 3.8 The process of homologous recombination and the products resulting from the double cross-over event between the wild type LSDV DNA and the pHSsgpt-VP7 plasmidFollowing digestion of the recombinant LSDV-VP7 DNA with *Bam* HI and hybridisation with the VP7 and pHSsgpt DIG-labelled probes, fragments of sizes 7.9 Kb and 6.268 Kb are expected if only recombinant LSDV is present. If parental wild type LSDV resides with the recombinant, an extra fragment of 11.5 Kb will be obtained.

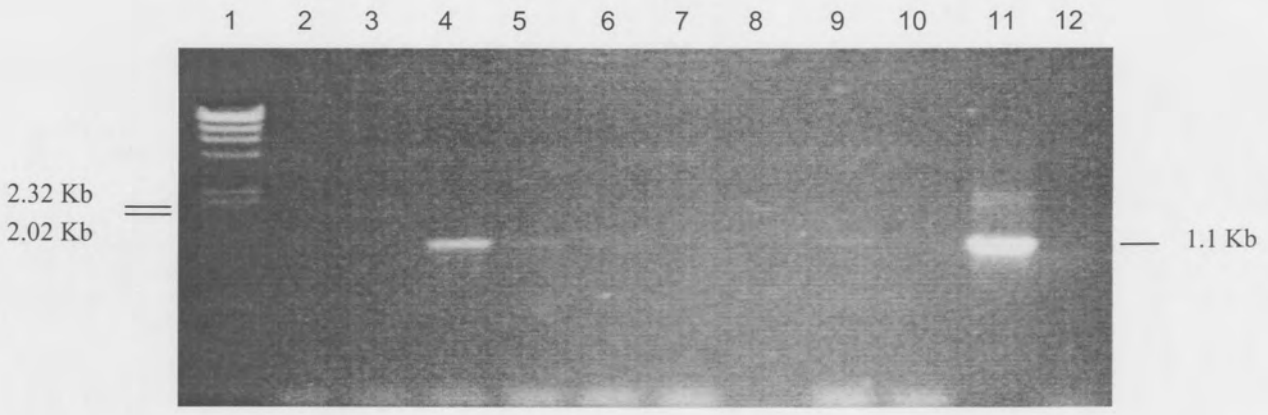

Fig 3.9 Agarose gel electrophoretic analysis of PCR products amplified from several candidate recombinant viral clones with primers Son2a and Son2b. Lane 1 contains the DNA molecular weight marker λII. Relevant sizes are indicated in Kb. Bands, identical in size to the 1.1 Kb fragment observed in the positive control reaction (lane 11), can be visualised in lanes 4-7, 9. No bands are visualised in lane 10, containing the reaction with wild type LSDV DNA template, or in lane 12 containing the negative control reaction.

3.3.4 Preparation and titration of a LSDV viral stock

One of the recombinant plaques, identified via PCR (section 3.3.3), was randomly chosen and amplified to yield a viral stock (described in section 3.2.11). The titre of the viral stock was determined (section 3.2.12) and found to be 2×10^7 pfu/ml.

3.3.5 Confirming the presence of recombinant virus via PCR

Viral DNA was isolated from the viral stock and tested, by means of PCR, for the presence of the VP7 gene, wt LSDV and the presence of LSDV (section 3.2.14). The pairs of primers utilised as well as the sizes of the expected PCR amplification products are listed in Table 3.2 (section 3.2.14). The PCR amplification products that resulted were visualised following 1% agarose gel electrophoresis (Fig 3.10). A 1.159 Kb band, amplified by primers Son 2a and Son 2b and shown in lane 2, confirmed the presence of the VP7 gene in the recombinant viral DNA. This band is the same size as the band amplified from the pBS-T7 positive control plasmid (lane 4). No bands were amplified from the wt LSDV control utilising these primers (lane 3). A 474 bp band was amplified, utilising primers Harry and Sally, from both the viral stock DNA and the wt LSDV DNA (lanes 5 and 6, respectively). This showed that, although the viral stock contained recombinant virus, it was contaminated with wt LSDV virus. Theoretically, primers Harry and Sally should have yielded a band of 2.959 Kb if the recombinant LSDV template was present. This band was not observed in lane 5. Previous experience (Dr W. Fick, personal communication) has indicated that these primers have difficulty amplifying such a large DNA fragment from a recombinant LSDV template. However, amplification of a 1.8 Kb amplicon with these primers from the control pHSsgpt DNA (lane 7) was easily achieved. The presence of LSDV in both the viral stock and the wt LSDV stock was confirmed by the amplification of a 166 bp band in lanes 8 and 9, respectively, by primers OP48 and OP49. No contamination occurred during PCR preparation, as no bands were visible in lane 10, containing the negative control reaction.

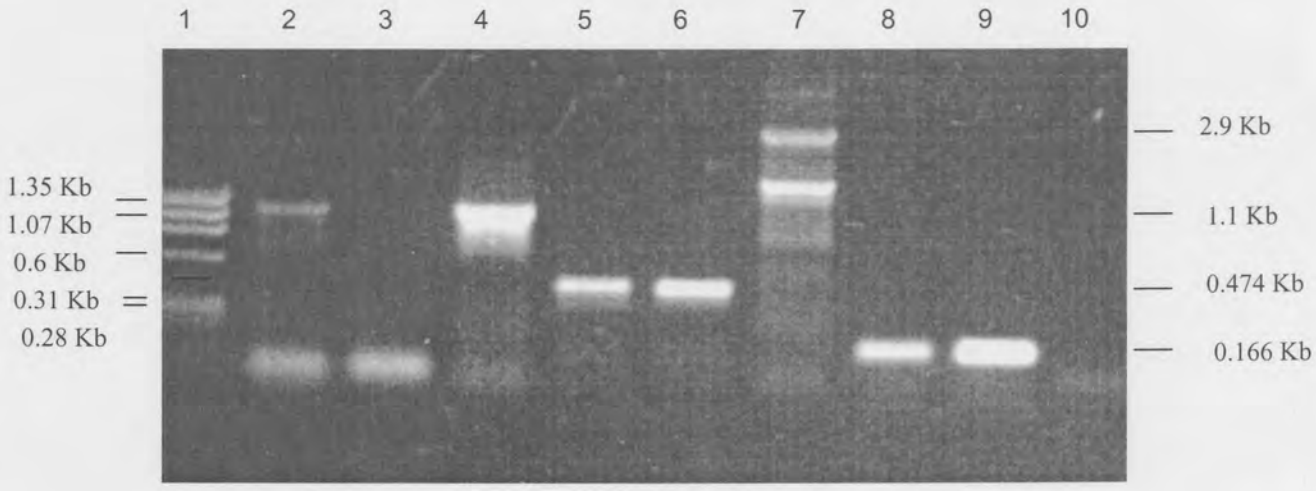

Fig 3.10 Agarose gel electrophoretic analysis of PCR products amplified from recombinant LSDV DNA and wild type LSDV DNA with several primer pairs. Lane 1 represents DNA molecular weight marker ϕX. Relevant sizes are indicated in Kb. PCR of the recombinant LSDV DNA with primers Son 2a and Son 2b resulted in the amplification of a single 1.1 Kb band (lane 2), identical in size to that amplified by the positive control (lane 4). No DNA fragments were amplified from the wild type LSDV DNA with these primers (lane 3). The presence of diffuse bands, approximately 100 bp in size, indicate the presence of excess PCR components (nucleotides, primers etc) in the PCR reactions. A band of 474bp was amplified from both the recombinant and wild type LSDV DNA lanes 5 and 6, respectively) with primers Harry and Sally. These same primers also amplified a 2.9 Kb band from the pHSsgpt control DNA (lane 7). Primers OP48 and OP49 amplified a fragment of 166bp from both the recombinant and wild type LSDV DNA (lanes 8 and 9, respectively). No amplification products are observed in lane 10, harbouring the negative control PCR reaction.

3.3.6 Determining the presence of recombinant LSDV DNA via a Southern blot

In order to confirm the presence of recombinant virus in the viral stock, a Southern blot procedure was performed. The presence of the foreign VP7 gene in the LSDV viral genome can be detected by blotting LSDV viral DNA onto a nylon membrane and hybridising it with specific DIG-labelled probes.

Recombinant LSDV-VP7 viral DNA, wt LSDV viral DNA and genomic LT DNA were each digested with the restriction endonuclease *Bam* HI (section 3.2.16). The contents of each reaction were loaded in replica and separated via electrophoresis (section 3.2.16). The resultant 0.6 % gel can be visualised in Fig 3.11. The agarose gel was utilised for a Southern blot procedure (section 3.2.17) and the nylon membrane, containing the transferred DNA, was cut into two pieces. Each piece of nylon membrane contained a replica of the fixed DNA. One membrane was hybridised with probes λII, ϕX and VP7 while the other was hybridised with probes λII, ϕX and pHSsgpt (section 3.2.17), resulting in Figures 3.12a and 3.12b, respectively. As no bands can be detected in lanes 3 and 4 (Fig 3.12a), it is clear that the VP7 probe did not bind to either the putative recombinant viral DNA or the wt LSDV DNA. It could be that the labelled VP7 probe was not sufficiently denatured prior to hybridisation. However this was not found to be the case as the positive control (nylon membrane onto which denatured pBS-T7 DNA had been spotted), included in the hybridisation reaction, exhibited a positive blue/purple precipitate after detection (results not shown). As the PCR results in section 3.3.5 indicated the presence of recombinant virus in the viral stock, the absence of a hybridisation signal could be due to the fact that the quantity of digested viral DNA loaded into the agarose gel was not sufficient to enable detection of the VP7 gene with the Southern blot procedure. The VP7 probe was not expected to hybridise to the genomic DNA of uninfected LT cells (Lane 5).

Following the hybridisation of the second membrane with the labelled pHSsgpt probe, Fig 3.12b resulted. The A2L and A3L sequences in the pHSsgpt plasmid bind to the homologous A2L and A3L regions of the LSDV genome. Recombinant and non-recombinant viral DNA will yield digestion products of different sizes (Fig 3.8). The faint 11.5 Kb bands, observed in lanes 3 and 4, indicated the presence of wt LSDV DNA in both the viral stock and wt LSDV viral stock. As the presence of recombinant LSDV-VP7 in viral stock has been confirmed (section 3.3.5), it is clear

that this entity is in the minority, with the overwhelming majority being wt LSDV. It was thus concluded that the quantity of digested viral DNA loaded into the 0.6 % agarose gel was not sufficient to detect the presence of the additional 7.9 Kb and 6.268 Kb recombinant bands in lane 3. The pHSsgpt probe did not bind to any sequences from the uninfected LT genomic DNA (lane 5), ruling out the possibility of probe non-specific binding.

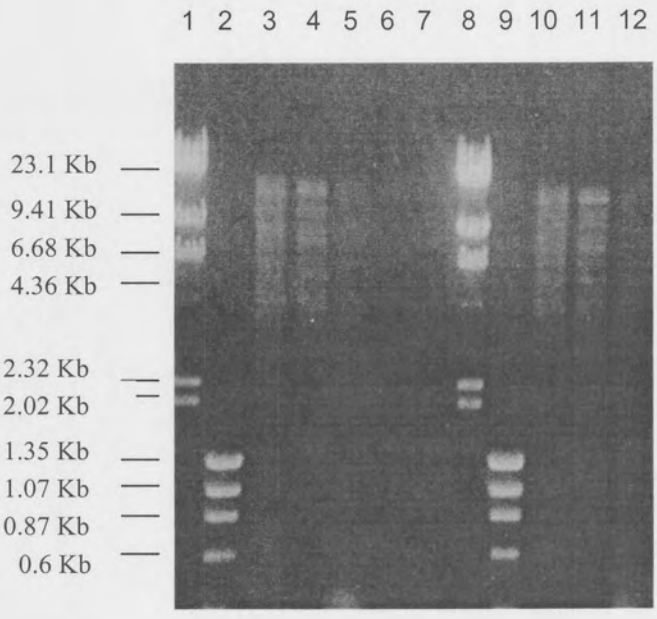

Fig 3.11 Agarose gel (0.6%) analysis of recombinant LSDV-VP7 viral DNA, wild type LSDV DNA and genomic LT DNA, each digested with restriction enzyme *Bam* HI. Lanes 1 and 2 contain the DNA molecular weight markers λII and ϕX, respectively. Relevant sizes are indicated in Kb. Lane 3 contains the digestion products of recombinant LSDV-VP7 viral DNA, while digestion products of the wild type LSDV viral DNA can be observed in lane 4. Less intense are the fragments resulting from the digestion of genomic LT DNA with *Bam* HI (negative control in lane 5). The samples loaded into lanes 1-5 were also loaded, in identical order gel (loaded in replica), into lanes 8-12 of the. No samples were loaded in lanes 6 and 7.

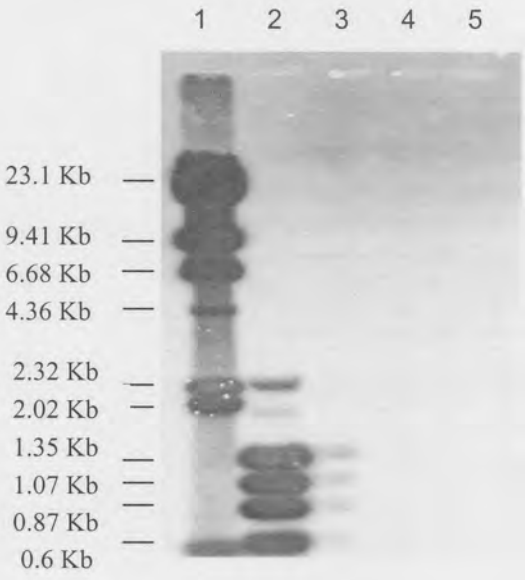

Fig 3.12a

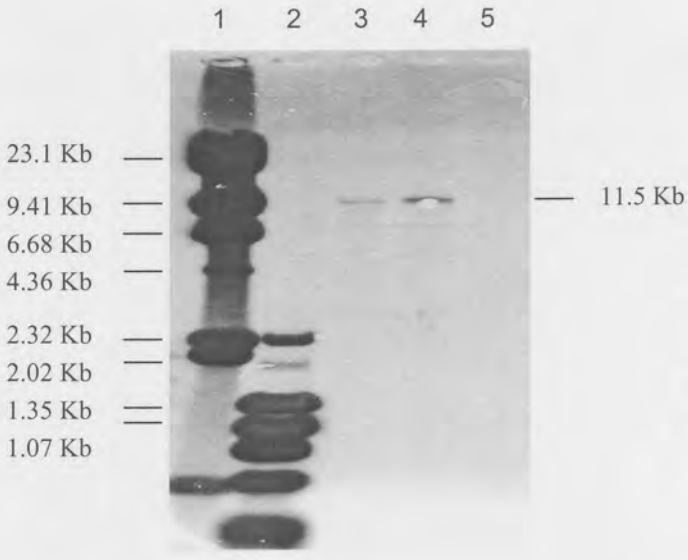

Fig 3.12b

Fig 3.12a Southern blot results obtained following hybridisation of one half of the gel depicted in Fig 2.11 with DIG-labelled λII, ϕX and VP7 probes. Lanes 1 and 2 contain the bands of the λII and ϕX DNA markers, respectively, detected following hybridisation of the DIG-labelled λII and ϕX probes. Relevant sizes are indicated in Kb. Two unexplained bands, 2.4 Kb and 2 Kb, can also be visualised in lane 2. No bands are visualised in lanes 3, 4 or 5 containing the recombinant LSDV-VP7, wild type LSDV and genomic LT DNA, respectively. The faint 1.35, 1.07, 0,87 and 0.6 Kb bands observed in lane 3 are because of sample overflow from lane 2.

Fig 3.12b Southern blot results obtained following hybridisation of the second half of the gel depicted in Fig 2.11 with the DIG-labelled λII, ϕX and pHSsgpt probes. Lanes 1 and 2 contain the bands of the λII and ϕX DNA markers, respectively, detected following hybridisation of the DIG-labelled λII and ϕX probes. Relevant sizes are indicated in Kb. The two unexplained bands, 2.4 Kb and 2 Kb, can be observed in lane 2. Hybridisation of the DIG-Labelled pHSsgpt probe resulted in the detection of an 11.5 Kb band in both lanes 3 and 4, containing the recombinant LSDV and wild type LSDV viral DNA, respectively. No bands were detected in lane 5.

3.3.7 Detection of expressed VP7 protein via a Western blot

The sensitive Western blot protocol was utilised in order to detect VP7 protein expression.

Proteins were isolated from infected LT cells at various times post-infection, as described in section 3.2.18. Proteins were isolated from wt LSDV infected and uninfected LT cells to serve as negative controls. As the protocol in section 3.2.19 states, 1/3 of each of these samples was electrophorised on a 12% polyacrylamide gel and stained in Coomassie Brilliant Blue staining solution to visualise the separated proteins (Fig 3.13). The 39 KDa band containing the VP7 protein was absent from lanes 2-5, containing the proteins isolated from infected LT at time intervals 24, 48, 72, 96 hours p.i respectively. This, however, does not mean that the protein is not expressed. Vos (2001) also failed to visualise a VP7 mt 177 protein, expressed in FBT cells, on a SDS-PAGE gel following Coomassie blue staining, but was able to detect its presence by radioactive protein labelling and SDS-PAGE. It can be observed that the intensity of the protein bands in lanes 2-5 gradually decreases. This can be explained by the fact that as the time interval p.i increases, the severity of the CPE on the cells increases proportionately. The quantity of proteins isolated from the decreasing number of harvested LT cells thus diminishes as time progresses. The absence of the 39 KDa protein in wt LSDV-infected and uninfected LT cells (lanes 6 and 7, respectively), was expected.

The same protein samples utilised above as well as a positive control, consisting of the whole cell fraction of Sf9 insect cells infected with a VP7-expressing baculovirus recombinant (donated by Q. Meyer) were electrophorised on a 12% SDS-PAGE gel. This gel was subsequently used in a Western blot analysis (section 3.2.20). The primary antibody, raised in guinea pigs against the whole cell fraction of Sf9 insect cells infected with an AHSV-9 VP7 baculovirus recombinant (Meiring, 2001), bound to all the proteins of the positive control (Fig 3.14, lane 7), as expected. An intense 39 KDa protein band, containing the VP7 protein, was clearly visible in lane 7 but not present in infected cells harvested at 24, 48, 72 and 96 hrs post-infection (lanes 1-4). As expected, the protein was also absent from cells infected with wt LSDV and uninfected LT cells (lanes 5 and 6, respectively).The 39 KDa band, visible in a protein sample harvested from uninfected LT cells, is most likely a result of the accidental overflow of proteins from well 7. Also a result of overflow are the bands

observed in lane 8 containing the rainbow protein size marker. The faint bands observed in lanes 1-5 are possibly the result of non-specific binding of the primary antibody to proteins isolated from wt LSDV. Some homology must thus exist between the mammalian-host LSDV proteins and the insect-host baculovirus proteins. As the Western blot procedure was not able to detect VP7 protein expression in LT cells infected with the stock virus, it was concluded that the VP7 protein was either not expressed or synthesised in quantities too small to detect utilising this procedure.

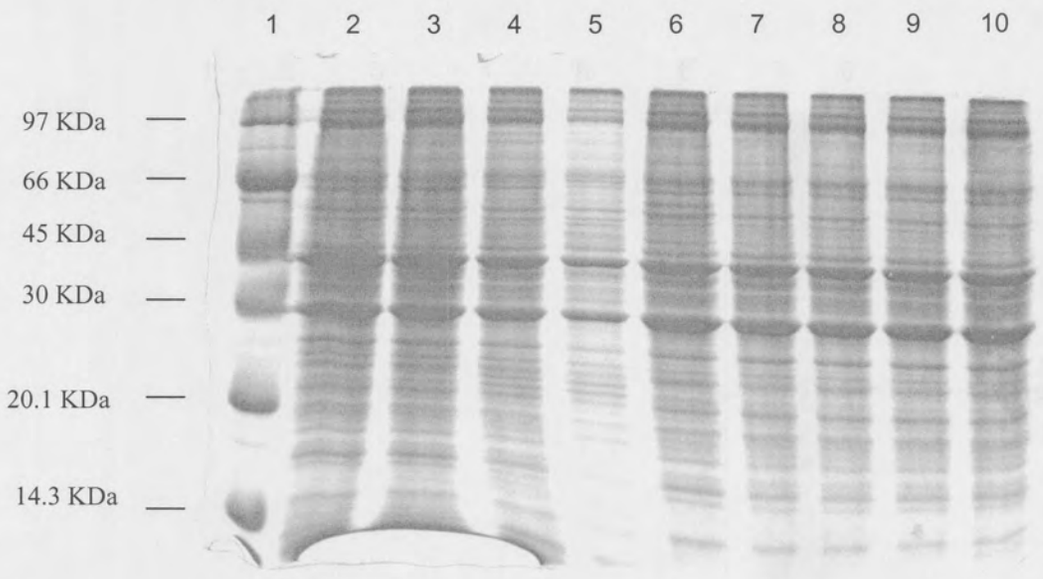

Fig 3.13 12% SDS-PAGE analysis and Coomassie blue staining of proteins isolated from recombinant LSDV-VP7 infected LT cells at various time intervals post infection. Lane 1 contains the Rainbow™ coloured protein molecular weight marker (Amersham Pharmacia) as a protein size marker. Relevant sizes are indicated in KDa. Lanes 2-5 contains proteins samples isolated 24, 48, 72 and 96 hours post infection, respectively. Lane 6 contains proteins harvested from wild type LSDV infected LT cells at 72 hours p.i, while lane 7 harbours a protein sample from uninfected LT cells. Lanes 8-10 contain protein samples irrelevant to this study.

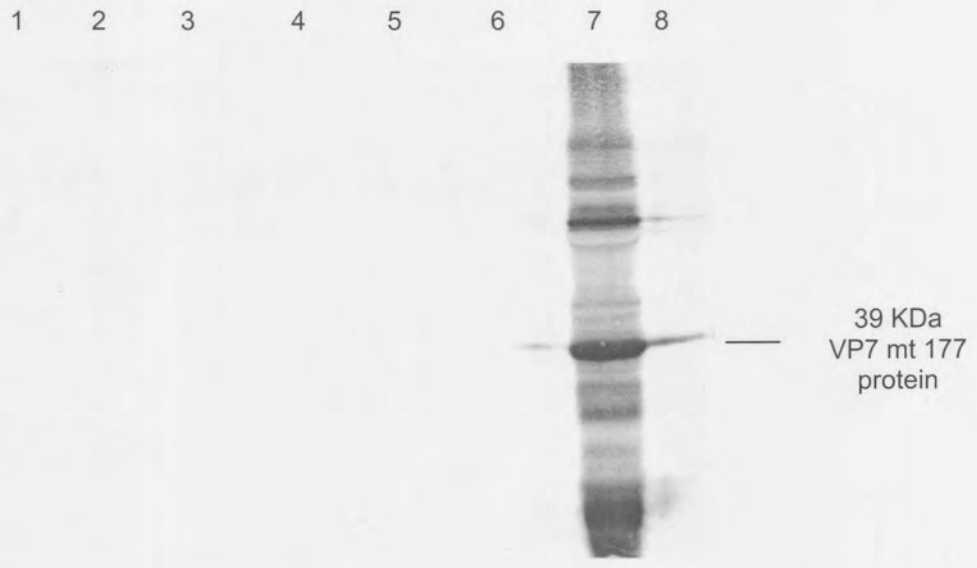

Fig 3.14 Determination of the presence of VP7 in protein samples isolated from LSDV-VP7 infected cells at various times post infection by means of the Western blot procedure. Lanes 1-4 contain proteins isolated from infected LT cells, 254, 48, 72 and 96 hours p.i, respectively. Lane 5 contains proteins harvested 72 hours p.i from cells infected with wt LSDV, while lane 6 contains proteins harvested from uninfected LT cells. Within lane 7 are proteins harvested from Sf9 insect cells infected with a VP7 baculovirus recombinant that served as a positive control. Lane 8 contains the Rainbow marker as a protein size marker, whose faint bands are not visible in Fig 3.15

3.3.8 Purification of viral stock

In order to proceed with the LSDV-VP7 recombinant every possible effort was made to eliminate the residual wt LSDV present in the viral stock. Only the supernatant, obtained from infected cells, was utilised for the infection of new cells under selective conditions. The supernatant contains mostly single budded viruses as opposed to the cells, which contain a large number of wt LSDV aggregates. The hypothesis is that by using only viruses present in the supernatant the possibility of infecting cells with a single recombinant virus is heightened (Wallace, personal communication). In order to break apart virus aggregates present in the supernatant, the supernatant was sonicated for 15 min and passed through a 0.45 μm filter. These steps were repeated three times in an effort to obtain a viral stock containing 90% recombinant virus (Wallace, personal communication). To obtain a homologous viral suspension, individual viral plaques were picked and passaged several times under selective medium. While these attempts were made to eliminate the residual wt LSDV, the recombinant LSDV-VP7 was also lost (PCR results with VP7-specific primers Son 2a and Son 2b not shown). Wt LSDV survived and continued to produce CPE in the presence of selective medium.

3.4 DISCUSSION

Some research involving LSDV (Neethling strain) as a live viral vector delivery system has already been completed. Obtaining high levels of foreign gene expression from LSDV would promote this vectors use as a delivery system. Consequently a unique bi-directional LSDV promoter was characterised and cloned into a carefully constructed transfer vector (Fick & Viljoen, 1999). A LSDV recombinant, with the AHSV-9 VP7 gene under the control of the early element of the bi-directional promoter, was subsequently generated via a process of homologous recombination (Vos, 2001). Very promising preliminary results show the levels of VP7 expression and crystal formation in infected Fetal bovine testis (FBT) cells to be higher than in comparable systems (Vos, 2001). These results indicate that the LSDV live viral vector system, expressing heterologous proteins from the unique LSDV bi-directional promoter, may have an advantage over comparative viral delivery systems. This study has focused specifically on the cloning of the AHSV-9 VP7 gene under the control of the late element of the bi-directional promoter in order to assess the strength of the two promoters and determine which of the promoters is suitable for high level expression of foreign, heterologous proteins, in the case of this study VP7.

In this chapter the construction of the recombinant transfer vector pHSsgpt-VP7 as well as the attempts to generate a recombinant LSDV-VP7 virus have been described. The effects of placing a foreign gene under the control of the late transcriptional element pA7L in a recombinant LSDV delivery system were also investigated. This was accomplished by following the strategy outlined below.

The donated plasmids pBS-T7 in T7 and pHSsgpt were digested with various restriction enzymes in order to obtain the gene of interest (VP7) and linearise the transfer vector, respectively. The insert and vector DNA molecules due to their non-complementary sticky-ends were blunt-ended by means of the Klenow reaction to facilitate cloning. Although the possibility of vector religation was reduced by dephosphorylating the pHSsgpt transfer vector, the efficiency of the blunt-ended cloning procedure was low. After numerous cloning and transformation attempts, tentative recombinant bacterial clones were tested by means of restriction enzyme analysis. A single recombinant clone was identified and the correct orientation of its insert confirmed by restriction enzyme digestion. The recombinant transfer vector,

pHSsgpt-VP7, was subsequently used in the generation of an LSDV-VP7 recombinant virus via the process of homologous recombination (as described in the introduction to this chapter). Following three rounds of selection against the wt LSDV in the presence of mycophenolic acid, viral plaques were picked and tested for the presence of recombinant LSDV-VP7 virus via PCR. Several plaques that contained recombinant virus were identified. The plaque which yielded the most intense positive amplification band was amplified in lamb testis cells to yield a high titer viral stock. The presence of recombinant virus in this viral stock was reconfirmed via PCR and the presence of parental wt LSDV was also detected. To determine whether the VP7 gene had indeed been inserted into the LSDV genome, a Southern blot procedure was performed. Results indicated the presence of wt LSDV genomic DNA but no indication of the presence of the VP7 gene in the LSDV genome. The failure of the VP7 specific probe to bind and yield a positive hybridisation signal could be explained by one of two hypotheses. Either the quantity of the digested LSDV DNA loaded into the agarose gel utilised for the Southern blot procedure was insufficient to allow for the detection of a small quantity of recombinant viral DNA, or there was simply no recombinant viral DNA present. If no recombinant DNA was present, the amplification of VP7-specific bands via PCR could be explained by the presence of small quantities of residual transfer plasmid carried over from the initial transfection procedure. This might lead one to the conclusion that the VP7 gene had never integrated into the LSDV genome but was amplified from the pHSsgpt-VP7 transfer plasmid extracted with the LSDV genomic DNA. However, as the virus had been put through several rounds of single plaque selection, this possibility seems unlikely. The possible recombinant was further investigated in the hope that the negative Southern blot results were due to an insufficient quantity of digested LSDV DNA being loaded onto the agarose gel.

Although it was clear that the majority of the viruses in the viral stock were wt, it was reasoned that the titer of the recombinant virus might still have been sufficient to detect VP7 protein expression. This assumption, however, was not realised. A Western blot was performed in order to detect possible small quantities of VP7 protein. PAGE and Coomassie Blue staining did not enable visualisation of the 39 KDa VP7 protein among the proteins isolated from infected LT cells. However, Vos (2001) also failed to visualise the VP7 protein by following the above protocol, but could detect expression of the 39 KDa protein following radioactive-labelling of the proteins expressed in infected cells and SDS-PAGE. In this study it was opted to utilise the non-radioactive Western blot procedure to determine whether expression

of the VP7 gene from the late promoter pA7L could be detected. Although this antibody-based protocol is known to be very sensitive, VP7 protein expression was not detected in LSDV-VP7 infected LT cells at various time intervals.

As no translation of the VP7 gene could be detected in the infected LT cells, it was assumed that the titer of the recombinant virus, if indeed it was present, was too low to enable detection of the VP7 protein. In order to increase the titer of the recombinant virus every effort was made to eliminate the wt LSDV present in the viral stock (Wallace, personal communication). However, the wt virus persisted even after several rounds of selection in the presence of mycophenolic acid. During this purification procedure, the LSDV-VP7 virus was lost.

In 1998, during a selection procedure, Dr Fick also lost a LSDV recombinant into which the VP2 gene had been cloned under the control of the late pA7L promoter. It has been suggested that the recombinant LSDV (type SA-Neethling) viruses are unstable due to the foreign gene being inserted into the viral thymidine kinase (TK) gene (Wallace & Viljoen, 2001). However, insertion of the marker gene Lac Z, also under the control of the late promoter pA7L, into another site in the genome as in this study, also resulted in the loss of the LSDV recombinant during subsequent selection steps (Fick, personal communication). It seems that although expression of a foreign gene from the early pA7R promoter was promising (Vos, 2001) LSDV recombinant, which contain foreign genes under the control of the late pA7L promoter, are unstable and are lost during the selection procedure.

Residual wt LSDV persisting during the selection procedure was not only encountered in this study but has also been a problem for other investigations (Fick, 1998; Vos, 2001). The notion that the LSDV viral vector system is a difficult system to work with is further enhanced by the fact that the LSDV host cells (LT and FBT) are primary cell lines, difficult to obtain and work with. Primary LSDV cell lines are not available commercially and can only be obtained at certain time periods from Onderstepoort Veterinary Research Institute (OVI). These cells are also only viable in cell culture for a finite number of generations, making them difficult to work with.

Researchers at Onderstepoort have, despite the obstacles mentioned above, managed to successfully generate and select stable, homogenous LSDV (type SA-Neethling, vaccine strain) recombinants expressing the *Escherichia coli* β-galactosidase (lac Z) reporter gene (Wallace & Viljoen, 2001). This gene was placed

under the control of the vaccinia virus 7.5 K early/late promoter and inserted into the viral thymidine kinase (TK) gene. Recombinants were selected, as in this study, by utilising the *E.coli* guanine phosphoribosyl transferase (gpt) gene as the dominant positive selectable marker. This research shows that with the use of different poxviral promoters and possibly different insertion sites, the generation of homogenous LSDV recombinants without the presence of wt LSDV is indeed possible. Thus the use of LSDV as a vector in recombinant vaccine production is still a viable option.

Much research has been done on the modified vaccinia ankara (MVA) viral strain as a recombinant vaccine vector. This virus is a severely attenuated and host-range restricted vaccinia virus strain (Moss, 1996). MVA was derived by more than 500 passages in Chicken embryo fibroblast (CEF) cells, during which multiple deletions and mutations occurred in the viral genome (Meyer, Sutter & Mayr, 1991). This resulted in the loss of the host range and host immunoregulatory genes (Antoine *et al.,* 1998; Blanchard *et al.,* 1998). Thus MVA can only replicate efficiently in CEF as well as Syrian baby hamster kidney (BHK-21) cell lines (Carroll & Moss, 1997). The virus is non-virulent in laboratory animals, including suckling and irradiated mice, as well as in immunosuppressed people (Wyatt *et al.,* 1998) and no host-range revertants of MVA have ever been reported.

It has been observed in most mammalian cells that the replication is incomplete due to defective virus assembly (Sutter & Moss, 1992). However, both early and late gene expression are unhindered and the expression of foreign genes is at least as efficient as the more virulent vaccinia virus strains (Sutter & Moss, 1992; Wyatt *et al.,* 1995). The first MVA recombinants constructed the T7 RNA polymerase (Wyatt *et al.*, 1995). MVA recombinants have subsequently been used to immunise mice against a number of diseases including influenza (Sutter *et al.*, 1994) and parainfluenza type 3 (Wyatt *et al.*, 1996; Durbin *et al.*, 1998).

Efforts at the UP Genetics department are thus now being focused on the modified vaccinia ankara (MVA) virus as a live viral vector system. Very promising are the facts that recombinant MVA can easily be generated without the presence of residual wt MVA (Staib *et al.*, 2000) and established cell lines such as Syrian baby hamster kidney cells (BHK) and rabbit kidney cells (RK-13) can be utilised to propagate MVA recombinants (Carroll & Moss, 1997). Although MVA has already been utilised to express HIV-1 antigens (as discussed in the Literature review), using it to deliver

chimeric VP7, into which specific HIV-1 epitopes have been inserted, is a novel strategy.

A viral system, such as MVA, that could circumvent the need for countless rounds of selection to eliminate residual wt parental viruses from recombinant stocks and could utilise established cell line would be ideal to use as a recombinant viral vector delivery system.

CHAPTER 4

CONCLUDING DISCUSSION

In this study, the HIV-1 subtype C strain Du 151 epitope-equivalent sequences ALDSWK and RVLAIERYLKD, were inserted into position 177 of the AHSV-9 VP7 protein. The construction of recombinant baculoviruses allowed for a high level of expression and consequently characterisation of the chimeric VP7 proteins. Although the immunogenicity of the ALDSWK and RVLAIERYLKD amino acid sqeeunces was not determined in this study, the availability of large quantities of the chimeric VP7 proteins obtained here will enable further immunological studies.

In addition to the uncertainty of whether the HIV-1 subtype C strain Du 151 epitope equivalent sequences were indeed epitopes, capable of inducing an immune response, it is unclear whether the AHSV-VP7 crystalline particles are an adequate epitope-presentation system. As the VP7 system is a novel system, not previously tested, the presentation of immunogenic epitopes on the VP7 protein surface may not result in the induction of an immune response. This has prompted the construction of chimeric VP7 proteins, into which the well-characterised subtype B epitopes ELDKWA and RILAVERYLKD, have been inserted into position 177 (Meyer, MSc thesis in publication). The induction of anti-ELDKWA and RILAVERYLKD neutralising antibodies by these chimeric VP7 proteins will confirm the suitability of the AHSV-9 VP7 crystalline particles as an epitope presentation system.

The generation of recombinant LSDV via homologous recombination being a time consuming process, one could argue that the construction of a LSDV recombinant expressing the chimeric VP7 gene (containing the ALDSWK or RVLAIERYLKD or both sequences) would have been more prudent than a LSDV recombinant expressing the native VP7 protein (Chapter 3). The recombinant LSDV delivery vector, expressing the chimeric VP7 protein, would thus have been available as soon as the antigenic status of the epitope-equivalent sequences ALDSWK and RVLAIERYLKD was determined (Chapter 2). However, a chimeric VP7 gene was not available when the project was

initiated. The construction of a chimeric VP7 transfer vector would therefore have taken a considerable amount of time to construct and would not have been wise to undertake considering the epitope status of ALDSWK and RVLAIERYLKD had not been determined. If these sequences were not found to be epitopes, a recombinant LSDV vector delivering them would thus be deemed useless. In addition, the expression studies done by Vos (2001) only involved the native VP7 protein. As the original aim of Chapter 3 was to compare the expression levels obtained from the early (pA8R) and late (pA7L) LSDV promoters, a comparative study should have involved an identical native VP7 protein with the promoter, under which the VP7 gene was cloned, being the only variable. This would have ensured that a chimeric VP7 protein would not affect the resultant expression levels obtained.

The results obtained in Chapter 3 indicate that the LSDV is not the ideal vector system for the delivery of heterologous foreign genes, such as VP7, into target cells. The generation of viral recombinants via the process of homologous recombination and the subsequent rounds of selection that are needed to eliminate wild type virus are only a few of the obstacles encountered when utilising this system. The loss of yet another LSDV recombinant, containing the VP7 gene under the control of the late promoter pA7L, has now focused efforts at the UP genetics Dept. on MVA as a promising live recombinant viral vector for the design of an HIV-1 vaccine.

Regardless which of the LSDV or MVA systems are utilised for the delivery of the chimeric VP7 protein, the VP7 protein will have to present the appropriate HIV-1 subtype C Th and CTL epitopes, in addition to the neutralising epitopes, in order to induce an effective immune response against HIV-1. The CTL epitopes will function to induce the much-needed cytotoxic cellular immunity, while the Th and neutralising epitopes will prime the humoral immune response when introduced into the target cells (Ourmanov, Bilska et al., 2000). Which Th and CTL epitopes should be included in a candidate HIV-vaccine is specifically determined by the population for which the vaccine is intended. Different populations exhibit variable allele frequencies of various MHC-I and MHC-II alleles. In order to construct a HIV-1 subtype C vaccine for Southern Africa, the frequency of the various MHC I and MHC II within each of South Africa's ethnic groups should be elucidated (Sullivan, 2000). Epitopes with amino acid sequences that would

be recognised by a majority of the population, harbouring common alleles, should be included into a candidate vaccine (Goulder *et al.*, 2000).

Ideally, a preventative AIDS vaccine would provide complete protection against HIV infection (Mascola & Nabel., 2001). However, most effective vaccines do not provide sterilising immunity but prime the host immune system to contain the invading pathogen after initial infection. Once infection occurs, because the HIV genome integrates into the host cell DNA, it may not be possible to completely eliminate the virus. Thus in the case of HIV-1, vaccine-induced immunity could contribute to the effective control of HIV-1 replication, leading to protection against disease progression and a reduction in the transmission of HIV-1.

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