UNIVERSITY OF CAPE TOWN

Assessing TMV as an immunogenic particle: The expression of HIV-1 subtype C V3 loop neutralizing antibody epitopes on the surface of TMV virions.

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CONTENTS

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ABSTRACT

In an attempt to establish a plant-based *Human immunodeficiency virus-1* (HIV-1) subtype C neutralizing antibody stimulating vaccine, a *Tobacco mosaic virus* (TMV) derived vector was used to express recognized HIV neutralizing antibody epitopes. These epitopes were expressed on the surface of the TMV coat protein, which served as an ideal means of antigen display. This model antigen display system was capable of assembling into multivalent, highly repetitive structures thereby displaying many copies of the attached epitope in the assembled virion. Three TMV-based vectors were acquired, which were essentially identical, differing only in the position at which they could accommodate a foreign protein fusion. These vectors allowed the display of a foreign peptide at the N-terminus, C-terminus or 60S-loop regions of the TMV coat protein, all of which protrude on the surface of the assembled virion. The HIV V3 loop is recognized as the principal neutralizing domain, and was the neutralizing epitopes displayed by the vectors. The epitope sequences used were derived from a cohort of infected individuals in Durban, South Africa, who displayed broad cross-neutralizing V3-specific activity towards heterologous viral strains. The recombinant viral vectors were shown to efficiently infect the host *Nicotiana benthamiana* plants and assemble into multivalent recombinant virion structures as observed by transmission electron microscopy. However, the level of coat protein expression was significantly dependent on the position of the coat protein fusion as either the levels of V3 epitope expressed or TMV coat protein was found to vary between the different vector types, confirmed by means of immunoblotting and enzyme-linked

immunosorbent assays. Immunogenicity analysis using a guinea pig model was used to assess the ability of the recombinant vectors to firstly establish a V3-specific immune response, and secondly to stimulate a virus-specific neutralization antibody response. As a result of time constraints only the C-terminal coat protein fusions were assessed in the guinea pig model. Inoculated guinea pigs displayed distinct and gradually increasing V3-specific immune responses after 2 boosts. Serum samples that displayed the strongest V3 peptide responses were then analyzed for their ability to neutralize HIV infection in HIV pseudovirion neutralization assays. Results for selected serum samples showed no HIV neutralizing activity above what could be recognized as background activity. Thus the candidate vaccine, although establishing a path for the assembly of a multivalent vaccine, failed in its attempt to stimulate a neutralizing antibody response. This study has nevertheless paved a direct path to the development of variations of this type of vaccine possibly using different and perhaps more effective epitopes for candidate vaccine purposes.

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
AMV	alfalfa mosaic virus
bp	base pair
cDNA	complementary deoxyribonucleic acid
CP	capsid protein
CTL	cytotoxic T-lymphocyte
DNA	deoxyribonucleic acid
dpi	days post inoculation
ELISA	enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
g	gram
HIV	Human immunodeficiency virus
HBsAg	Hepatitis B surface antigen
kDa	kilo Dalton
LA	Luria-Bertani agar
LB	Luria-Bertani broth
LSBC	Large Scale Biology Corporation
ml	millilitre
NAb	neutralizing antibody
ng	nanogram
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PND	principal neutralizing domain
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SHIV	chimaeric simian/human immunodeficiency virus
TCLA	T-cell line adapted
TMV	Tobacco mosaic virus
µg	microgram
µl	microlitre
V3	third variable loop
VLP	virus-like particle
wt	wild type

CHAPTER 1

Introduction and Literature Review

1.1 INTRODUCTION

In the present day, vaccines are considered to be an indispensable means of protection against an array of potentially lethal pathogens. Throughout recent history, they have provided an unsurpassed level of protection against several lethal diseases including smallpox and more recently, polio.

In spite of the continued efforts in the battle against the *Human immunodeficiency virus* (HIV), infection remains rife and the epidemic continues to spread. Current statistics of HIV infection highlight the problem the medical world is faced with. As of the end of 2004 the estimated number of individuals living with HIV was approximately 40 million worldwide. Approximately two thirds of these people are located in sub-Saharan Africa. During 2004, an estimated 5 million new HIV infections occurred internationally. This translated to 14000 infections per day, 95% of which occurred in developing countries (http://www.niaid.nih.gov/factsheets/aidsstat.htm).

An untreated person will almost certainly fail to successfully combat an HIV infection, as infection is accompanied with an early immune dysfunction, which limits control of the virus. During HIV infection the virus is directed towards those cells of the immune system presenting $CD4^+$ receptors at their surface (Popovic *et al.*, 1989; Zack *et al.*, 1990; Dalgleish *et al.*, 1991; Douek *et al.*, 2002; Lawson *et al.*, 2004; Oxenius *et al.*, 2004). Infection of these cells usually results in their lysis as a result of virus-induced and host immune system-induced consequences in response to infection, and a depletion of those cells required for the immune response results. As the various immune response pathways are interlinked, the

host's immune response is effectively crippled by the depletion of these cells. Most fatalities that arise from acquired immune deficiency syndrome (AIDS) are therefore a consequence of opportunistic infections, which are sufficient to kill the host due to the weakened immune system. An immune system which has been primed with an HIV vaccine however, would hopefully allow early recognition of the infecting virus and subsequent destruction of virus particles before they have an opportunity to become established within the host (Mascola *et al.*, 2000; Baba *et al.*, 2000; Letvin, 2002).

As no effective HIV vaccine is currently present, drug therapy for the treatment of HIV-infected people is the alternative to vaccination. Currently several highly effective drugs exist such as protease inhibitors (ritonovir, saquinovir), nucleoside-analogue reverse transcriptase inhibitors (AZT, lamivudine) and non-nucleoside reverse transcriptase inhibitors (nevirapine) (http://aegis.com/factshts/network/simple/combo.html). The primary drawbacks of this form of therapy are that it is very expensive, has unpleasant side effects and its use is generally exclusive to those who can afford it. In spite of the vast amounts of research, no form of drug therapy has to date been demonstrated to cure an HIV infection.

Vaccines have therefore been proposed as a means to prevent the virus from firmly establishing an infection within a previously uninfected person. Ideally, a vaccine directed against HIV should induce a neutralizing antibody (NAb) response, which would clear all detectable levels of HIV within the infected person (sterilizing immunity). Several such vaccine strategies have been tested but, despite this, a vaccine capable of stimulating a completely sterilizing immunity

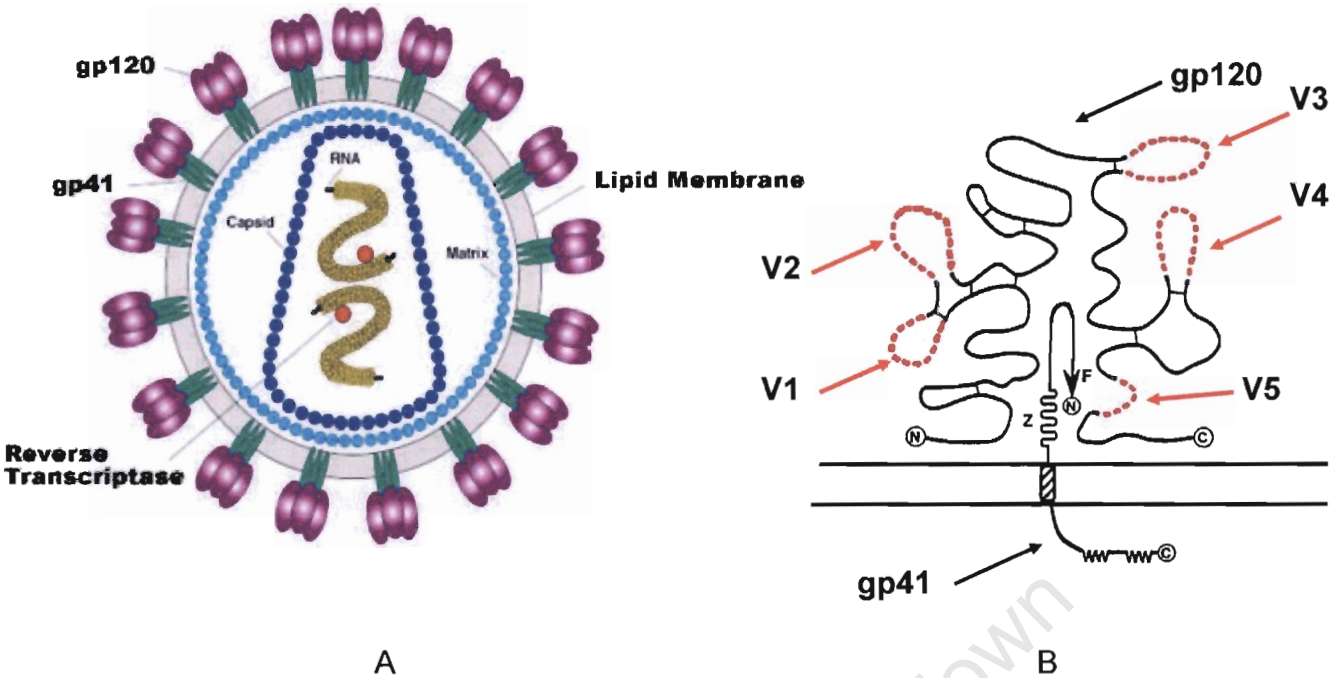
remains elusive (http://aidsinfo.nih.gov/vaccines/). The development of an effective HIV vaccine is regarded as one of the most important future prospects with respect to vaccine design. In the ongoing battle against HIV, numerous potential vaccine strategies have reached pre-clinical and even clinical human trial levels (http://www.hvtn.org/science/trials.html). However, these studies have generally not generated encouraging results with regard to the development of a safe and effective vaccine and specific reference is made here to the recent HIV envelope protein based (AIDSVAX) phase III vaccine trials by VaxGen, which appeared to offer no significant protective efficacy (Adis International Ltd, 2003; Anonymous, 2003).

1.2 HIV ENVELOPE GLYCOPROTEINS

HIV presents a 550 amino acid surface glycoprotein, gp120, at the exterior surface of the virion membrane (Figure. 1.1A) (Fields *et al.,* 1996). This surface glycoprotein is non-covalently associated with the transmembrane protein gp41 (Figure. 1.1B) and presents a binding domain for CD4 receptors found on the surface of T-lymphocytes, monocytes, macrophages, mature B-cells and dendritic cells. These gp120/41 associations form clusters of tri-molecular complexes on the virion membrane surface (Lu *et al.,* 1995; Chan *et al.,* 1997; Farzan *et al.,* 1998). Gp120 interaction with target CD4 receptors and chemokine co-receptors (CCR5/CXCR4) plays an important role during virus infection by initiating the fusion of the virion membrane to the host cell membrane, and thus allowing entry of the virus into the host cell (Dalgleish *et al.,* 1991; Deng *et al.,* 1996; Wyatt *et al.,* 1998 A). The HIV genome encodes the envelope glycoproteins as a single 160 kDa precursor (gp160), which is subsequently cleaved into the 120 kDa (gp120)

and 41 kDa (gp41) functional glycoproteins by the virus encoded protease. Thus during the membrane fusion process, if gp120 interaction with the CD4 receptors and chemokine co-receptors is considered as the initiation step in cellular infection, the activity of the gp41 transmembrane protein could be viewed as the functional unit that facilitates actual membrane fusion as follows (Chan *et al.*, 1998): free virions are assumed to be in a native or non-fusogenic conformation, in which the gp41 fusion peptide that is inserted into the target membrane is buried inside the trimeric complex; upon the CD4 and chemokine receptor binding by gp120, structural rearrangement alters the gp120/41 interaction and triggers gp41 to undergo structural rearrangement. The exposure of the gp41 fusion peptide accompanies the structural rearrangement; it is then inserted into the target cell membrane, which causes further structural rearrangement within the gp41 trimeric-coiled coil resulting in membrane apposition, which allows fusion of the virion and cell membranes.

The gp120 amino acid sequence contains 18 highly conserved cysteine residues, which are assumed to play a critical role in the structural organisation and functionality of gp120 (Tschachler *et al.,* 1990; Travis *et al.,* 1992; Chiou *et al.,* 1992; Fields *et al.,* 1996; Wyatt *et al.,* 1998). Structural modelling has revealed that this protein is divided into several conformation-dependent regions, through the formation of disulphide bonds between cysteine residues. These are required for cellular receptor binding (CD 4) (Figure. 1.1B) (Trkola *et al.*, 1996; Fields *et al.,* 1996; Hoffman *et al.*, 1999; Hung *et al.,* 1999; Wang *et al.,* 1999). Heterogeneity between individual HIV-1 isolates within gp120 has been localized to 5 variable loops (V1 – V5) of which 4 are cysteine-linked at each base (Starcich *et al.,* 1986).



A B

Figure. 1.1(A). Illustrates the position of the gp120 surface glycoproteins and gp41 transmembrane proteins on the suface of an HIV virion (www.aegis.com/topics/basics/hivandaids.html) ©1980, 2002. AEGiS. (B) Illustrates the tertiary structure of gp120 and displays the positioning of the variable loop structures (V1, V2, V3, V4, V5) (red arrows) as well as the non-covalent association with gp41 (derived from Fields *et al.,* 1996)

1.2.1 Immunogenicity of HIV envelope glycoproteins

Research has indicated that some antibodies directed towards envelope glycoproteins possess the ability to neutralize HIV infection (Weiss *et al.,* 1986; Schwartz, *et al.*, 1993; Muhlbacher *et al.,* 1999; Sattentau *et al.,* 1999; Collado *et al.,* 2000). The gp120 glycoprotein has been observed to elicit both a neutralizing (NAb) as well as a non-neutralizing antibody response during HIV infection (Wyatt *et al.,* 1998 B). The large majority of antibody responses raised against gp120 in HIV-infected people are found to be non-neutralizing (Burton, 1997; Burton *et al.,* 2000). Non-neutralizing antibodies are often directed against regions of gp120 that may be hidden in the assembled trimer (Wyatt *et al.,* 1998 B). For this reason it is believed that the humoral immune response during HIV infection is directed against viral debris and not the virus (Thiriart *et al.,* 1989; Burton, 1997; Parren *et*

al., 1997(A) and (B)). This deduction made by Burton (1997), is consistent with the kinetics of envelope processing and rapid turnover of infected cells. It is observed that only a small fraction of gp160 is processed into mature gp120 in infected cells (Willey *et al.*, 1988). Burton (1997) has suggested that during rapid turnover of infected cells, large amounts of gp160 are released. This should primarily target a strong initial immune response to gp160, which may suppress the immune response towards lower concentrations of the mature envelope (Trkola *et al.,* 1996 A). Alternatively, NAbs access the functional envelope glycoprotein complex and bind epitopes near the receptor or co-receptor binding sites (Wyatt *et al.,* 1998 B).

NAb responses *in vivo* can take months or years to develop (Morris, 2002), which would allow HIV infection to be firmly established in the host (Burton, 1997). The importance of priming B-cell memory NAb immune responses is clearly highlighted in a study conducted by Buckner *et al.,* (2004), who utilised HIV-envelope glycoproteins as immunogens to stimulate NAb responses in macaques. As expected, vaccinated macaques failed to develop a completely sterilizing immunity but were able to control plasma viraemia levels as a result of rapidly induced NAbs and remained disease free for the entire 3-year observation period. The unvaccinated control group also developed a NAb response to the challenge chimaeric simian human immunodeficiency virus (SHIV), although at a much slower rate. Animals in this group rapidly progressed to a diseased state and fatalities were observed within the observation period.

The need for a more rapid, B-cell primed memory response is inferred in a case where it is found that NAb responses in recently infected individuals do arise within

a few months following infection but this appears to exert a selective pressure that allows escape of neutralization-resistant mutants (Richman *et al.,* 2003). This then necessitates a repeat of the process of having to stimulate new NAbs, which are able to recognise the new predominant circulating neutralization-resistant mutants. Priming NAb B-cell memory thus does appear to have played a crucial role in controlling viral replication in the infected host.

The oligomeric form of gp120 is known to display a limited array of epitopes recognisable by neutralizing antibodies. The accessibility of these epitopes is better understood when observing the core structure of gp120 (Figure 1.2A). The gp120 core consists of 2 domains: an inner domain that faces the trimer axis and gp41, and the outer domain that is exposed on the surface of the trimer (Wyatt et al., 1998 B). There is also a third region referred to as the bridging sheet, which does not belong to either domain completely. In this core structure variability is also associated with the surface of the outer domain core proximinal to the V4 and V5 loops. Variability here is contributed by the A, B, C, D and E surface loops, which serve as potential N-linked glycosylation sites in the gp120 core in this half of the protein (Wyatt *et al.,* 1998 B). As most carbohydrate moieties are recognised as self to the immune system, this renders this region of gp120 less visible to immune surveillance and thus it is referred to as the silent face of the protein (Wyatt *et al.,* 1998 A, B). In addition to this, a neutralizing as well as a non-neutralizing face of gp120 has been identified (Figure. 1.2A). The neutralizing face is situated at the base of the trimeric complex and contains the co-receptor binding site, which is shielded by the V2 and V3 loops (Figure. 1.2B). The non-neutralizing

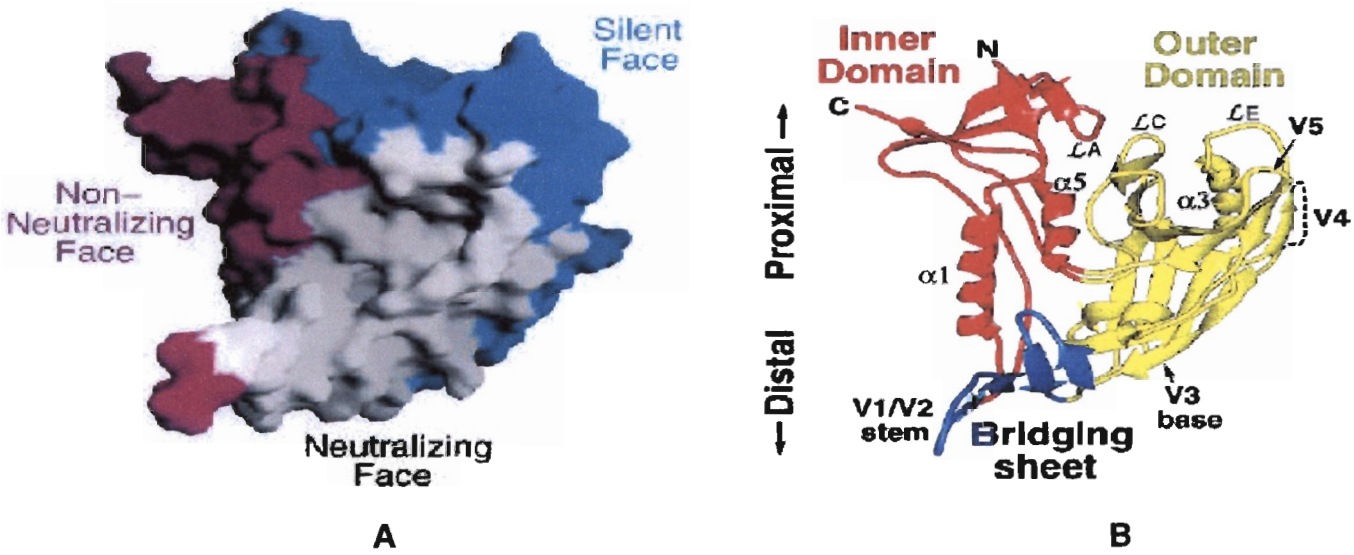


Figure. 1.2. This illustrates the core structure of the gp120 envelope protein. (A) Shows approximate locations of the faces of the gp120 core, defined by the interaction of gp120 and antibodies. The molecular surface accessible to neutralizing ligands is shown in white. The neutralizing face of the complete gp120 glycoprotein includes the V2 and V3 loops, which are found adjacent to the surface shown. The approximate location of the gp120 face that is poorly accessible on the assembled envelope glycoprotein trimer and therefore elicits only non-neutralizing antibodies is shown in magenta. The approximate location of an immunologically 'silent' face of gp120, which roughly corresponds to the highly glycosylated outer domain surface, is in blue. (B) This shows the ribbon diagram of the gp120 core seen from the perspective of CD4, and is oriented with the viral membrane at the top of the figure and the target cell membrane at the bottom. The inner gp120 domain is shown in red and the outer domain in yellow; the 'bridging sheet' is blue. The N and C termini of the truncated gp120 core are labelled, as are the positions of structures related to the gp120 variable regions V1–V5. The gp120 surface is implicated in binding to the CCR5 chemokine receptor. Images and permission for use kindly provided by Dr Richard Wyatt, Chief, Structural Virology Section, Vaccine Research Center/NIH, (Wyatt *et al.*, 1998B).

face has been identified to contain the CD4 receptor-binding site (Wyatt *et al.*, 1998 B).

Only a few target sites on gp120 have been recognised to act as NAb epitopes and these include the epitopes defined by the MAbs 2G12 and IgG1b12 (Burton, 1997). Epitope 2G12 binds to a region defined as a cluster of high-mannose type oligosaccharides proximal to the surface implicated with co-receptor binding near the base of the V3 loop on HIV-1 gp120 (Morris, 2002; Calarese *et al.*, 2003; Wang *et al.*, 2004; Li *et al.*, 2004). Epitope IgG1b12 has been identified to bind to the

region proximal to the CD4-receptor binding site. is located in the membrane proximinal part of the gp41 glycoprotein (Muster *et al.,* 1994). Gp41 has also been shown to contain broadly neutralizing ligands recognized by the 2F5 and 4E10 antibodies (Zwick *et al.*, 2001; McGaughey *et al.*, 2004; Cardoso *et al.*, 2005).

1.2.2 V3 Loop

The third variable loop (V3) in gp120, located between amino acids Cys301 – Cys336, is critical for defining co-receptor tropism of the virus (Fields *et al.,* 1996). V3 induces one of the most potent NAb responses able to bind both HIV virions and infected cells, and has thus been termed the principal neutralizing domain (PND) of the virus (D'Souza *et al.,* 1991; Javaherian *et al.,* 1991; Spear *et al.,* 1992, Rizzuto *et al.,* 1998, 2000). The potency of the immune response against V3 can be attributed to the important role it plays in cell tropism (detection of target cells), cytopathicity and fusogenicity (Hoffman et al., *1999*; Hung *et al.,* 1999; Hwang *et al.,* 1991; Wang *et al.,* 1999). As a consequence of these key functions attributable to V3 during the early stages of virus infection, the potential ability of V3-specific antibodies to neutralize virus infection is evident. Mediation of virus neutralization can proceed by either blocking the binding of gp120 to chemokine co-receptors or inhibiting virion entry at a step after virus attachment (Trkola *et al.,* 1996; Hill *et al.,* 1997). Such methods of neutralization have also previously been demonstrated in an assay where synthetic V3 peptides were capable of blocking HIV infection of primary lymphocytes and macrophages (Yahi *et al.,* 1994).

Several studies have revealed that V3 is a major determinant of chemokine receptor binding (Hoffman *et al.*, 1999, Trkola *et al.*, 1996). Deletion analysis has

demonstrated that V3-deleted forms of gp120 are unable to bind CCR5 receptors even when CD4 receptor binding has occurred at wild-type levels (Trkola *et al.,* 1996). Subsequent research by Cormier *et al.* (2000, 2001 and 2002) has revealed that the interaction between soluble gp120-CD4 complexes and CCR5 N-terminal sulfopeptides requires amino acid residues which primarily reside within the V3 stem and C4 regions of gp120. Residues within the V3 crown, on the other hand, have been shown to play an important role in soluble gp120-CD4 complex binding to cell surface CCR5, where they interact with the extracellular loop regions of CCR5. Thus entry into target cells is dependent on the subsequent interactions between gp120 and both the CD4 target receptor and the N-terminal domain of the CCR5 or CXCR5 co-receptors. Subsequent research has also suggested that specific residues within V3 may also play an equally important role in mediating the post-entry integration of viral DNA (Suphaphiphat *et al.,* 2003).

The binding of antibodies to V3 is found to interfere with gp120-CCR5 binding (Trkola *et al.,* 1996). The major function of CD4 receptor binding has been suggested to involve induction of conformational changes in gp120 that contribute to the exposure of the chemokine co-receptor binding site (Sattentau *et al.,* 1991; Bandres *et al.,* 1998). However, some clinical laboratory isolates of HIV no longer depend on CD4 for entry, requiring only chemokine receptors instead (Clapham *et al.,* 1992). Several HIV-2 strains have demonstrated an ability to infect CD4-negative cell lines. Clapham *et al.,* (1992) have suggested that chemokine receptors may represent the "*primordial obligate receptors*" and the use of CD4 receptors may have evolved to enhance chemokine receptor binding. Targeting an immune response towards V3 should therefore take preference to an immune

response directed towards CD4. A study by Reeves *et al* (1997), in which molecular clones with a CD4-independent tropism were used, demonstrated the critical residues required for CD4-independent tropism to reside within V3. Modelling studies on gp120 have suggested that the V2 and V3 loops, which reside opposite one another in the structure, may play a role in shielding the conserved elements of the chemokine receptor (Wyatt *et al.,* 1998 B). An immune response directed towards the chemokine receptor will therefore not always be completely possible, although it should be possible to block chemokine interaction by targeting an immune response towards V3.

1.2.3 V3 loop sequence conservation

In spite of V3 being a variable domain, relatively conserved domains within it have nevertheless been identified. These conserved domains reside within the crown of V3 (306-322) in HIV-1 isolates, whereas variable domains generally reside within the 10-15 amino acids occurring on either side of the crown (Spear *et al.,* 1994). The V3 monoclonal antibody 447-52D is recognized as one of the most broadly neutralizing antibodies and recognizes a 14 amino acid sequence that contains the GPxR core sequence residing at the crown of the V3 loop (Zolla-Pazner *et al.*, 2004; Conley *et al.*, 1994). Similarly the 58.2 monoclonal antibody is known to potently neutralize TCLA HIV-1 strains, which also recognises the V3 sequence that constitutes the crown of the V3 loop (HIGPGRAF) (Cabezas *et al.*, 2000; Dong *et al.*, 2004). Several studies have now demonstrated the cross clade neutralizing activity of both polyclonal and monoclonal antibodies to V3 loop against both primary and TCLA HIV isolates (Gorny *et al.*, 2002; Krachmarov *et al.*, 2001; Moore *et al.*, 1995; Conley *et al.*, 1994). The immunological significance of V3 is

highlighted in a study conducted by Spear *et al.* (1994) who analysed the relative contributions of V3-specific antibodies in the sera of HIV-infected individuals. Very high levels (30-40%) of the sera from HIV-infected persons that were capable of binding infected cells were V3-specific. This study also compared the ability of the entire gp120 protein and a V3 peptide to inhibit complement stimulated by sera isolated from infected persons: gp120 demonstrated a 92% capacity to inhibit complement compared to the central epitope of V3 which displayed an 82% capacity. This is very significant considering that V3 only constitutes 3% of the gp120 amino acid sequence.

Immune responses elicited by V3-C4 peptide chimaeric-based vaccines have also been shown to neutralize primary SHIV isolates. In a follow-up study, this peptide provided a partial protection against a SHIV-89.6 challenge in rhesus macaques (Liao *et al.,* 2000; Letvin *et al.,* 2001). Variations of V3-based vaccine candidates have also included Gag virus-like particles (VLPs), which present V3 epitopes fused to their subunit C-termini (Luo *et al.,* 1991; 1997). In these instances, assembled Gag VLPs have allowed the simultaneous presentation of several V3 peptides per VLP and have shown to stimulate NAb responses to serum of inoculated rabbits. The inoculation of the Gag VLPs alone (no V3 epitope presented) did not stimulate a similar NAb response. Humoral immune responses in these cases (stimulated by Gag VLPs) and others have been fairly type specific, very few demonstrating ability to recognize and neutralize heterologous isolates despite displaying several different V3 epitopes in tandem. Cytotoxic T lymphocyte (CTL) responses to these Gag-V3 VLPs were also analysed (Luo *et al.,* 1997). Splenocytes from mice inoculated with the Gag-V3 VLPs developed a broad CTL

response and lysed target cells displaying the V3 epitope contained in the VLPs used. Thus, despite the importance of the humoral immune response, it is evident that an effective vaccine should also stimulate the cellular arm of an immune response to be effective. The aforementioned studies specifically highlight the importance of choosing epitopes for a vaccine that induce an antibody response capable of neutralizing primary HIV/SHIV isolates and not only T-cell line adapted strains (TCLA) as indicated by several other studies (Gorny *et al.,* 1997, 1998; Spenlehauer *et al.,* 1998). These studies indicate more promisingly that NAb responses can be stimulated from individual V3 peptides. This suggests that the "correct" 3-dimensional or native context of the peptide in gp120 is not necessarily imperative for stimulating NAb responses.

The recent analysis of serum samples derived from a cohort of HIV-1 subtype-C infected South African sex workers was analysed (Bures *et al.,* 2002). Extensive cross neutralization between the serum samples and heterologous isolates has suggested the presence of shared neutralization determinants. Shared neutralization determinants in effect is desirable for the development of a vaccine as it suggests that a neutralizing antibody response will have a smaller antigenic diversity to overcome at a regional level. During the course of this study, serum derived from 2 isolates, Du151 and Du179 after less than 2 years of infection were capable of neutralizing a large number of both HIV-1 subtypes C and TCLA B subtypes to a level similar to that displayed by the serum of HIV-infected non-progressors. Sera from the subjects were also found to react strongly with the autologous V3 peptide and also showed a broad cross-reactivity with heterologous subtype C V3 peptides. V3-specific reactivity and neutralization activity of sera was

particularly strong for subjects Du151 and Du179. V3 epitopes from HIV isolated from these 2 individuals were selected for my study purposes.

1.3 VACCINE DESIGNS

Currently, several different vaccine types including DNA, protein subunit and live attenuated vaccines have been designed and tested against HIV in ongoing trials (Boyer *et al.*, 2000; Qui *et al.*, 1999; Morris *et al.*, 2000; Morris *et al.*, 2002; Bruce *et al.*, 1999). Protein subunit vaccines are deemed to be one of the safer alternatives as HIV-specific vaccines, with the ability to potentially stimulate an efficient immune response without posing the threat of reverting to a virulent form and causing disease as demonstrated by some live attenuated vaccine models (Barouch *et al.*, 2002; Arntzen, 1997).

Traditionally, protein subunit vaccines have been produced in mammalian cell cultures, bacteria and yeast. These processes call for the development of highly specialised facilities and equipment for production, purification and storage of the protein products. Additional complications may arise as the prokaryotic protein synthesis machinery does not require post-transcriptional or post-translational modification mechanisms such as glycosylation, and this may lead to such complications as incorrect protein folding (Marino, 1991). These complications and the expense have made this form of vaccine production an expensive process (Awram *et al.*, 2002) (Table 1.). This would severely limit access to treatments prepared by these methods by the most needy. The need for more cost-effective vaccines is highlighted annually by the approximately 2 million unnecessary deaths per annum in undeveloped nations (Langridge, 2000). These deaths occur as a

result of 20% of infants worldwide not being vaccinated against diphtheria, pertussis, polio, measles, tetanus and tuberculosis due to the sheer expense.

Table1. Comparison of production systems for recombinant human pharmaceutical proteins (Ma *et al.*, 2003)

System	Overall cost	Production timescale	Scale-up capacity	Product quality	Glycosylation	Contamination risk	Storage cost
Bacteria	Low	Short	High	Low	None	Endotoxins	Moderate
Yeast	Medium	Medium	High	Medium	Incorrect	Low risk	Moderate
Mammalian cell culture	High	Long	Very low	Very High	Correct	Viruses, prions and oncogenic DNA	Expensive
Transgenic animals	High	Very long	Low	Very High	Correct	Viruses, prions and oncogenic DNA	Expensive
Plant cell cultures	Medium	Medium	Medium	High	Minor differences	Low risk	Moderate
Transgenic plants	Very low	Long	Very high	High	Minor differences	Low risk	Inexpensive

1.3.1 Vaccine Production in Plants

Recent advances in molecular biology have provided a more cost-effective alternative for the bulk production of foreign proteins: this is the use of plants as bioreactors. This concept of using plants as a means of producing proteins for subunit vaccines was first introduced in 1992 by Charles Arntzen and co-workers (Arntzen *et al.,* 1997; Walmsley *et al.*, 2003), which has since given rise to the concept of molecular farming. The prospect of producing foreign proteins in plants is particularly attractive as plants can be grown locally and do not require highly-specific growth nutrients or conditions and facilities (Awram *et al.*, 2002). The production of foreign proteins within plants grown locally for vaccine purposes could also resolve the logistical and economic challenges of transporting the vaccine over long distances. It has been estimated by Evangelista *et al.* (1998) that the production of proteins in plants may be up to 50 times less expensive than in

E.coli. As indicated by Ma (2000), the prospect of producing vaccines within plants should, apart from lowering production costs of newly-developed vaccines, also reduce the costs of already-existing protein subunit vaccines. Several provisionally successful recombinant subunit vaccines have been produced in plants (Table. 2).

Table 2. Recombinant subunit vaccines produced in plants (Ma *et al.*, 2003)

Foreign protein expressed	Plant host	
Hepatitis B virus envelope protein	Tobacco	First vaccine candidate expressed in plants; third plant derived vaccine to reach clinical trials stage
Rabies virus glycoprotein	Tomato	First example of an "edible vaccine" expressed in edible plant tissue.
Escherichia coli heat-labile enterotoxin	Tobacco, potato	First plant vaccine to reach clinical trials stage
Norwalk virus CP	Potato	Second plant vaccine to reach clinical trials stage
Diabetes autoantigen	Tobacco, potato	First plant-derived vaccine for an autoimmune disease
Cholera B subunit	Tobacco, potato	First vaccine candidate expressed in chloroplasts
Cholera toxin B and A2 subunits, rotavirus enterotoxin and enterotoxigenic *E. coli* fimbrial antigen fusions	Potato	First plant derived multivalent recombinant antigen designed for protection against several diseases
Porcine transmissible gastroenteritis virus glycoprotein S	Tobacco, maize	First example of oral feeding inducing protection in an animal

Foreign protein expression systems in plants can be established in 2 main ways. The first involves the generation of transgenic plants, which involves the permanent insertion of the foreign gene of interest into the plant nuclear or chloroplast genome (Rigano *et al.*, 2005). Several antigens including the Hepatitis B and E virus surface antigen, rabies virus glycoproteins, Human papillomavirus (HPV) type 16 L1 major CP, rotavirus VP7 neutralizing protein, Escherichia coli heat-labile toxin, Foot and Mouth disease virus VP1 protein and many others have been expressed from either the nuclear or chloroplast genomes in transgenic

plants (Mason *et al.*, 1992; Ma *et al.*, 2003; Mc Garvey *et al.*, 1995; Varsani *et al.*, 2003; Wu *et al.*, 2003; Kang *et al.*, 2004; Sun *et al.*, 2003; Daniell *et al.*, 2005).

The major alternative is the use of recombinant virus-derived plant expression vectors, which allow the hopefully high-level transient expression of a foreign gene of interest. Both these different techniques have their own advantages and disadvantages. A comprehensive knowledge of plant tissue culture is required when generating transgenic plants. This process is drawn out and labour-intensive, and generally transgenic plants are unable to reach the high expression levels attainable by viral expression vectors. Recombinant viral vectors on the other hand, have the potential to rapidly produce high levels of the protein in the infected plant within 1-4 weeks of inoculation (Awram *et al.*, 2002). These high expression levels are facilitated by the ability of the virus to spread systemically throughout the infected plant and produce the foreign protein in all infected cells. Optimal protein expression levels can be determined by the protein, the viral vector used, and the type of plant to be infected.

1.4 PLANT VIRAL VECTORS

Potential plant viral vector candidates used in previous research include the isometric viruses Cowpea mosaic virus (CPMV), Tomato bushy stunt virus (TBSV) and Caulimovirus based vectors (Awram *et al.,* 2002; Usha et al., 1993; Joelson *et al.,* 1997; De Zoeten *et al.,* 1989). Helically-assembling plant viruses can also be added to this repertoire of plant viral vectors, and these include the tobamovirus-based vectors, Alfalfa mosaic virus-derived (AMV) vectors, Potexvirus-based vectors as well as Potyvirus based vectors (Awram *et al.*, 2002; Yusibov et al.,

1997; Sugiyama *et al.*, 1995, Marusic *et al.*, 2001). *Tobacco mosaic virus* (TMV), a member of the *Tobamoviridae*, is one of the more prominent vectors. Historically TMV has been one of the most well studied viruses.

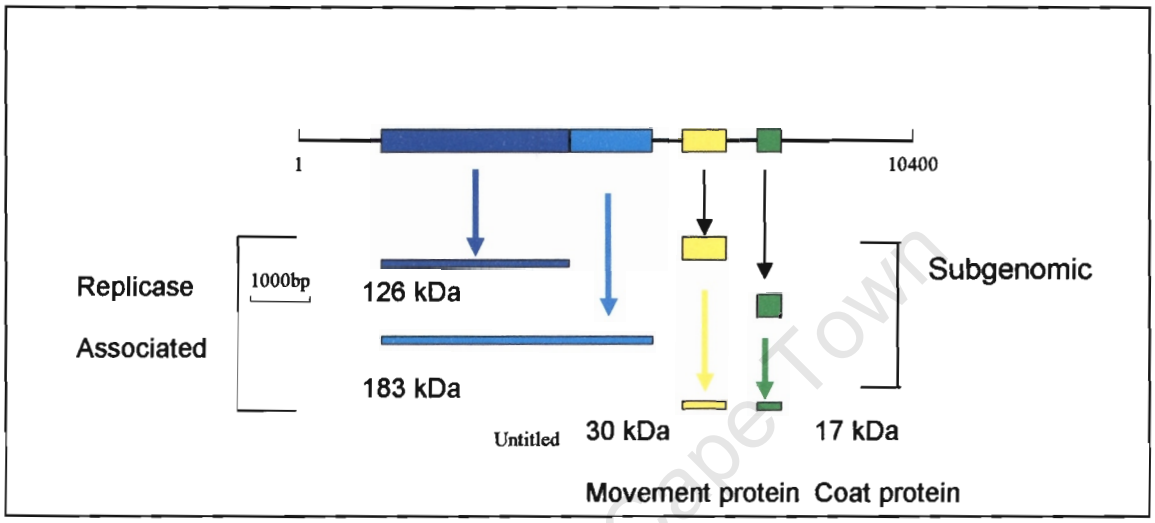


Figure. 1.3. Illustrating all open reading frames within a linearized representation of the TMV genome (http://www.biosource-technologies.com/genead.html)

1.4.1 TMV Plant Viral Vectors

Several investigators have exploited the potential of the TMV CP (CP) in vaccine development to generate CP hybrids that display a variety of foreign epitopes including those from malaria (*Plasmodium*), HIV gp120, Influenza virus and Murine hepatitis virus (Turpen *et al.*, 1995; Sugiyama *et al.*, 1995; Koo *et al.*, 1999). Numerous other investigators have also succeeded in stimulating the production of significant levels of peptide-specific antibodies using these TMV CP hybrids (Oscherwitz *et al.*, 1999; Fitchen *et al.*, 1995; Staczek *et al.*, 2000, Wu *et al.*, 2003).

TMV is a plus-sense single-stranded RNA (ssRNA) virus whose genome consists of 6395 nucleotides, and contains only 4 open reading frames (ORF) (Figure. 1.3) (Goelet *et al.*, 1982). TMV expresses 3 genes; a 126kDa as well as a 183kDa protein are expressed from the 5' end of genomic RNA by means of a leaky translational stop codon directly (Yusibov *et al.*, 1999). These 2 proteins are known to form a replicase complex required for efficient replication (Ishikawa *et al.*, 1991). Other proteins translated from subgenomic RNAs during viral replication include a 30kDa movement protein, which is required to facilitate passage of the virus particles from cell to cell via the plasmodesmata (Wolf *et al.*, 1989) and a 17.5kDa CP, which is required for the long distance movement of the virus within and between plants.

The N- and C- termini of the CP are known to protrude on the exterior surface of the TMV virion, which make them ideal for epitope presentation (Figure. 1.4C) (Namba *et al.,* 1989). Epitopes fused to the purified CP can be presented in an aggregated or particulate form as the CP monomers at low pH are capable of self-assembly by aggregating into rod-like particles independent of viral RNA, of which a typical wild-type TMV virion contains 2130 monomers (Figure. 1.4A and B) (Butler, 1984).

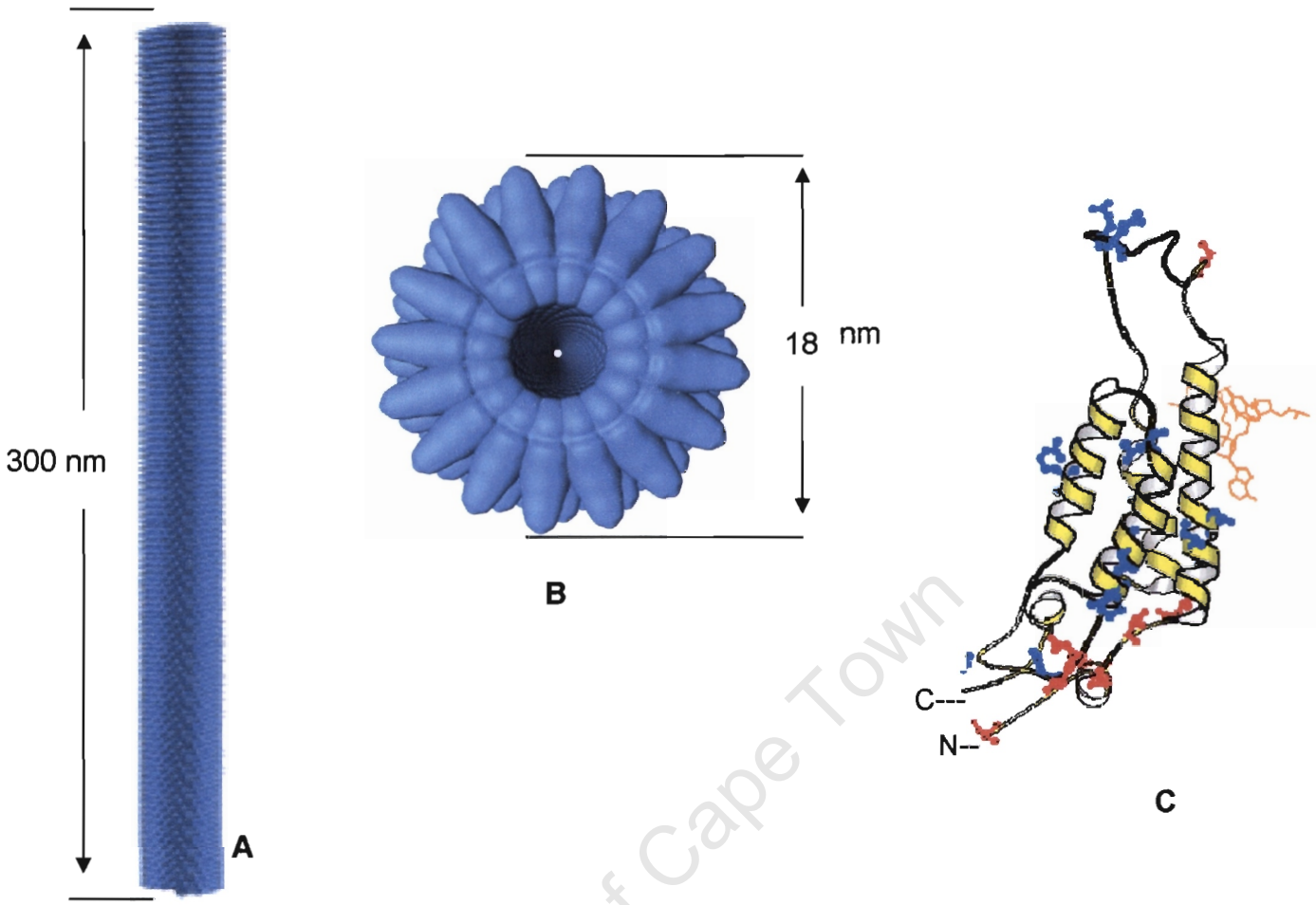


Figure. 1.4(A) and (B) display 3-dimensional data of the assembled rod-shaped TMV virion (www.ppws.vt.edu/ ~sforza/tmv/tmv.html) All content and images ©Copyright Peter Sforza, 2001; (C) Shows a ribbon diagram of a TMV coat protein monomer; the N- and C-termini lie next to one another and are exposed at the surface of the assembled rod (http://www.biologie.uni-hamburg.de/b-online/e35/7.htm) © Peter v. Sengbusch. Images were kindly provided by the indicated researchers.

The TMV CP has been shown to be strongly immunogenic in the aggregated state or as virions, and probably contains T-cell epitopes (Dr K.E. Palmer, LSBC, personal communication). This property is quite noteworthy, as recombinant "display" particles can almost certainly strongly enhance the immunogenicity of a fused epitope. Enhanced V3 peptide immune responses have been demonstrated by means of using a highly immunogenic Hepatitis B surface antigen (HBsAg) to present the HIV-1 MN V3 peptide (RIHIGPGRAFYTTKN). The HBsAg/V3 fusion

elicited humoral immune responses against both viruses within 3-6 weeks, which peaked at 6-12 weeks and remained stable for 25 weeks. This observed response was far superior in comparison to the use of gp160 peptide alone, which only induced a slow and low-titred anti-V3 response at 12 weeks post-inoculation. This demonstrated the ability of an immunogenic carrier protein to act as an adjuvant enhancing the weak anti-V3 response (Fomsgaard *et al.,* 1998).

Properties that promote the use of the TMV as a vector for gene expression within plants include the fact that the 17.5 kDa TMV CP is capable of being expressed at high levels in the infected plant. The viral CP has already been shown to accumulate to levels as high as 10% of the infected dry leaf mass (Fraser *et al.,* 1987; Yusibov et al., 1999). This ability to rapidly produce high levels of protein has been credited to the fact that TMV is an RNA virus. TMV has also displayed a rapid systemic spread within a range of host plants (Donson *et al.,* 1991). Replication is limited to the cytoplasm and not the nucleus and thus the absence of nuclear integration coupled to viral replication kinetics enables rapid production of proteins (http://www.biosourcetechnologies.com/genead.html). A property of immense significance is that the TMV RNA genome can be used to make cDNA clones that are easily modified to allow foreign gene expression (Shivprasad *et al.*, 1999; Donson *et al.*, 1991). Another important fact about TMV is that it has a very broad host range, and one of its primary host species - *Nicotiana tabacum* or tobacco, is also one of the highest biomass and soluble protein-producing crops in the world (Yusibov *et al.*, 1999). As TMV is a plant virus it does not pose the threat of infecting mammalian cells and will therefore be non-pathogenic and non-toxic to a vaccinated individual.

As TMV is an RNA virus, this could deter some who consider using it as a potential vector because of the low fidelity of RNA polymerases (replicase) (Yusibov et al., 1999). A study conducted by Kearney *et al.* (1993) examined the accumulation of errors within a TMV-derived vector over a period of 6 months and 10 passages from plant to plant. Of the viral RNA isolated and sequenced, no mutations were identified. Similar results obtained with another RNA virus based vector, TBSV, corroborate the finding that these vectors are potentially quite stable, as the vector retained the correct epitope sequence through a series of 6 sequential passages (Joelson *et al.,* 1997). Despite the stability, however, all recombinant viruses used in this study were found to lose their insert eventually. This may work in favour of use of the vector, as "escapes" can almost be guaranteed to revert to "wild-type" on serial transmission preventing expression of foreign gene in other nearby crops.

It has been established that peptides are efficiently presented to the immune system in repetitive arrays (Broekhuijsen *et al.,* 1986; Lo-Man *et al.,* 1993; Lowenadler *et al.,* 1990; Zheng *et al.,* 1993). Such displays allow for more efficient cross-linking of antigen-specific immunoglobulins on B-cells, which leads to B-cell proliferation and antibody production. A study conducted by Oscherwitz *et al.* (1999) has compared the ability of immunogens containing single as well as 8 tandemly repeated copies of V3 to elicit an immune response. The study revealed that the tandemly repeated immunogen was capable of inducing an approximately 30-fold greater antibody response compared to those elicited by the monomeric immunogen. Similarly Yusibov *et al.* (1997) used a recombinant TMV expression system, which utilised the AMV CP to express V3 peptide from HIV. The recombinant, self assembled CP when injected into mice stimulated a neutralizing

humoral immune response, which resulted in up to 80% neutralization of the HIV-1/MN isolate.

1.5 PROJECT OUTLINE AND OBJECTIVES

The aim of this study was to develop a peptide-based vaccine capable of inducing a NAb response to HIV-1 subtype C, the predominant subtype circulating in sub-Saharan Africa. The V3 PND was identified as the epitope of choice to stimulate the NAb response. A TMV-based expression system was selected as a means of efficiently expressing, producing and presenting the V3 loop epitope. The foreign V3 loop epitopes were fused to different positions in the TMV CP, to potentially allow the display of these epitopes on the surface of the assembled TMV virion: theoretically, this could allow display of approximately 2000 copies of the V3 peptide on the outer TMV virion surface. Both TMV vector systems (CP fusions) and V3 peptides have shown promise in their individual respects as an efficient expression system and a potent Nab-stimulating immunogen in the varying contexts discussed earlier. The combination of these 2 factors could therefore present a potent HIV NAb stimulating vaccine candidate that if successful, could form part of a combination therapy regimen. As the proposed TMV based vaccine candidate's production is plant based, this study aimed to enable a cost-effective means of vaccine production, which is one of the primary aims for vaccine production in developing nations.

CHAPTER 2

Construction of Recombinant TMV Chimeras

2.1 INTRODUCTION

A primary concern when investigating a potential vaccine is whether the selected epitope/s presented to the immune system are capable of stimulating an adequate immune response (capable of clearing an infection), be it humoral or cellular. The aim of this study was to stimulate the humoral arm of the immune response and determine whether a significant HIV NAb response could be stimulated with a recombinant carrier particle. In particular, an epitope capable of stimulating a broadly cross-NAb response to the predominant HIV-1 subtype circulating in sub-Saharan Africa was most desirable. As discussed earlier (chapter 1 page 11), the HIV-1 subtype C gp120 V3 (PND) epitope was identified as the epitope of choice to elicit this immune response.

NAb responses were recently analysed in a cohort of South African sex workers infected with HIV-1 subtype C (Bures *et al.*, 2002). Sera from the subjects were found to react strongly with the autologous V3 peptide and also showed a broad cross reactivity with heterologous subtype C V3 peptides. V3-specific reactivity and neutralization activity of sera was particularly strong for subjects Du151 and Du179. In some instances Du151 serum was shown to react more strongly with heterologous V3 sequences than their autologous sera. Prior research on Du151 and Du179 subject-derived isolates revealed that Du151 isolates used the CCR5 receptor while Du179 isolates were dual tropic, using both CCR5 and CXCR4 as co-receptors (van Harmelen *et al.*, 2001). Dual tropism is an uncharacteristic trait of subtype C viruses and is in general attributed to an increase in positive charges from arginine residues within V3. Sequencing has shown that the increased positive charge in this instance arose due to the presence of a histidine residue at

position 290 (which is located near the base of the V3 loop), which increased the overall positive charge to +6. This is characteristic of CXCR4 co-receptor tropism (van Harmelen *et al.*, 2001). Another uncharacteristic trait of V3 from DU179 isolates identified in this study is the 34 amino acid length as opposed to the normal 35 amino acid length. V3 peptides derived from the Du151 and Du179 isolates therefore serve as contrasting candidates for stimulating NAb responses and both were selected for this study. For the purposes of this study, HIV isolates derived from subjects Du151 and Du179 will be referred to as Du151 and Du179 respectively.

As mentioned previously, the TMV CP can accommodate the ligation of epitopes at various positions that allow for the presentation of the foreign epitope on the surface of the assembled virion. This ability to manipulate the RNA virus genome arises due to the fact that the viral genome can be presented in a cDNA cloning vector form, which later is *in-vitro* transcribed to facilitate viral infection. Three TMV-based cloning vectors were used in this study. They allowed for the fusion of V3 epitopes to the N-terminus, C-terminus or in the 60S loop regions of the TMV CP. Traditionally, TMV CP fusions have not exceeded approximately 20 amino acids in length, suggesting that larger epitopes would sterically hinder TMV virion assembly and may affect virion solubility (Sugiyama *et al.,* 1995; Yusibov et al., 1997; Koo et al., 1999; Staczek *et al.,* 2000; Wu *et al.,* 2003). To prevent this possibility, truncated forms of the V3 PND were fused to the TMV CPs.

This chapter describes the assembly and construction of recombinant V3-presenting TMV vectors from the base TMV vectors provided by Large Scale Biology Corporation (LSBC).

2.2 MATERIALS AND METHODS

2.2.1 Assembly of V3 epitopes

HIV-1 subtype C V3 PND amino acid sequences derived from patients Du151 and Du179 were kindly provided by Dr Lynn Morris (National Institute for Communicable Diseases, Johannesburg, South Africa).

Du151: N- NNNTRKSIRIGPGQTFYATGEII -C (23 amino acids)

Du179: N- GNNTRKSIRIGPGQAFYTNHII -C (22 amino acids)

These epitopes were cloned into the appropriate LSBC TMV-based cloning vectors (pLSB2295, pLSB2109, pLSB2296; Dr K.E. Palmer, LSBC, CA, USA) at the *NcoI* (*NcoI* = 5'-CCA TGG-3') and *NgoMIV* (*NgoMIV* = 5'-GCC GGC-3') restriction enzyme cloning sites, resulting in fusion of the epitope at either the N-terminus (pLSB2295) or C-terminus (pLSB2109) or to the 60S loop (pLSB2296) (Appendix B 2, 4, 6).

The epitope-encoding sequences were therefore modified as follows, each epitope-encoding sequence averaging approximately 70 base pairs (bp) in length. The oligonucleotides synthesized by the Oligonucleotide Synthesizing Service of the Department of Molecular and Cell Biology, University of Cape Town could not exceed 40 bp in length if they were to retain any relative degree of accuracy. To facilitate the synthesis of the V3 epitope encoding sequence I therefore decided to use a PCR assembly technique derived from Stemmer *et al.* (1995).

In addition, Du151 and Du179 sequences needed to be synthesized to have an *NcoI* site at the 5'-encoding end and an *NgoMIV* site at the 3'-encoding end to allow for easier cloning.

As mentioned previously, the V3 PND-encoding sequence was truncated to avoid steric hindrance of virion assembly. Appropriate truncation of each epitope was accomplished by removing the first and last 4 amino acids from both Du151 and Du179 encoding sequences resulting in 2 overlapping epitope-encoding regions highlighted in Figure 2.1.

Du151: (a)

5'-CC ATG GGT AAC AAC AAC ACC CGG AAG AGC ATC CGG ATC GGC CCC GGC CAG ACC TTC TAC GCC ACC **GCC GGC-3'**

N N N T R K S I R I G P G Q T F Y A T

Du151: (b)

5'-CC ATG GGT CGG AAG AGC ATC CGG ATC GGC CCC GGC CAG ACC TTC TAC GCC ACC GGC GAG ATC ATC **GCC GGC-3'**

R K S I R I G P G Q T F Y A T G E I I

Du179: (a)

5'-CC ATG GGT GGC AAC AAC ACC CGG AAG AGC ATC CGG ATC GGC CCC GGC CAG GCC TTC TAC ACC **GCC GGC-3'**

G N N T R K S I R I G P G Q A F Y T

Du179: (b)

5'-CC ATG GGT CGG AAG AGC ATC CGG ATC GGC CCC GGC CAG GCC TTC TAC ACC AAC CAC ATC ATC **GCC GGC-3'**

R K S I R I G P G Q A F Y T N H I I

Figure 2.1 This shows the 4 different V3 loop epitope encoding sequences that were synthesized for cloning into the pLSB vectors. The highlighted areas indicate the regions where the Du151, Du179 (a) and (b) epitopes differ as a result of the epitope truncations. From the highlighted areas on each epitope it is clear that the (a)-epitopes are C-terminal truncations while the (b)-epitopes are N-terminal truncations

2.2.1.1 PCR Assembly Strategy

The High Fidelity PCR System (Roche) was used as per vendor-directed protocol (www.roche-applied-science.com/pack-insert/1732641a.pdf). The PCR assembly strategy involved dividing the epitope-encoding sequence into 4 overlapping oligonucleotides for each epitope (Appendix C(I)). Oligonucleotides (A) and (C) encompassed the forward strand, while (B) and (D) encompassed the reverse strand. These oligonucleotides then via a 2 stage PCR indicated below (Figure 2.2) were annealed ((A to B) and (C to D)) and then amplified in a 2 stage PCR (Figure 2.2). The indicated intermediate PCR products are shown in figure 2.2. The intermediate oligonucleotides generated, i.e. the elongated oligonucleotides A and D, annealed at their complementary regions and completed the entire epitope-encoding sequence. Thus at the end of stage 1, a pool of nucleotides existed that included those encoding the correct epitope sequence as well as others that resulted from the annealing of oligonucleotides B and C, which clearly cannot be amplified. A second stage of PCR was employed to more accurately define and concentrate the required epitope-encoding sequence. This second stage produced more specific product by using the external forward and reverse oligonucleotides/primers A and D.

2.2.1.2 PCR compositions:

For the stage 1 PCR a 25 pmol/μl working stock dilution of each oligonucleotide was made and the 4 oligonucleotides (A, B, C, D) combined in equimolar amounts to form the oligonucleotide mixture. Each reaction was carried out in a final volume of 50μl. Reactions were carried out using

components of the High Fidelity PCR System (Roche), which included 1x high fidelity buffer, 2.5mM $MgCl_2$, 0.125mM dNTP mix, 1.75 units high fidelity enzyme mix, 2μl (25pmol/μl) oligonucleotide mixture, 36.5μl dH_2O (See reaction temperature profile Appendix C(II)).

The stage 2 reactions were also carried out in a final volume of 50μl. Reactions were carried out using components of the High Fidelity PCR System (Roche), which included 1x high fidelity buffer, 2mM $MgCl_2$, 0.125mM dNTP mix and 1.75 units high fidelity enzyme mix. Reactions also contained 1μl oligonucleotides A and D, 36.25μl dH_2O, and 1.25μl stage 1 PCR product (which constitutes a 1/40 dilution of stage 1 product, see reaction temperature profile Appendix C(II)).

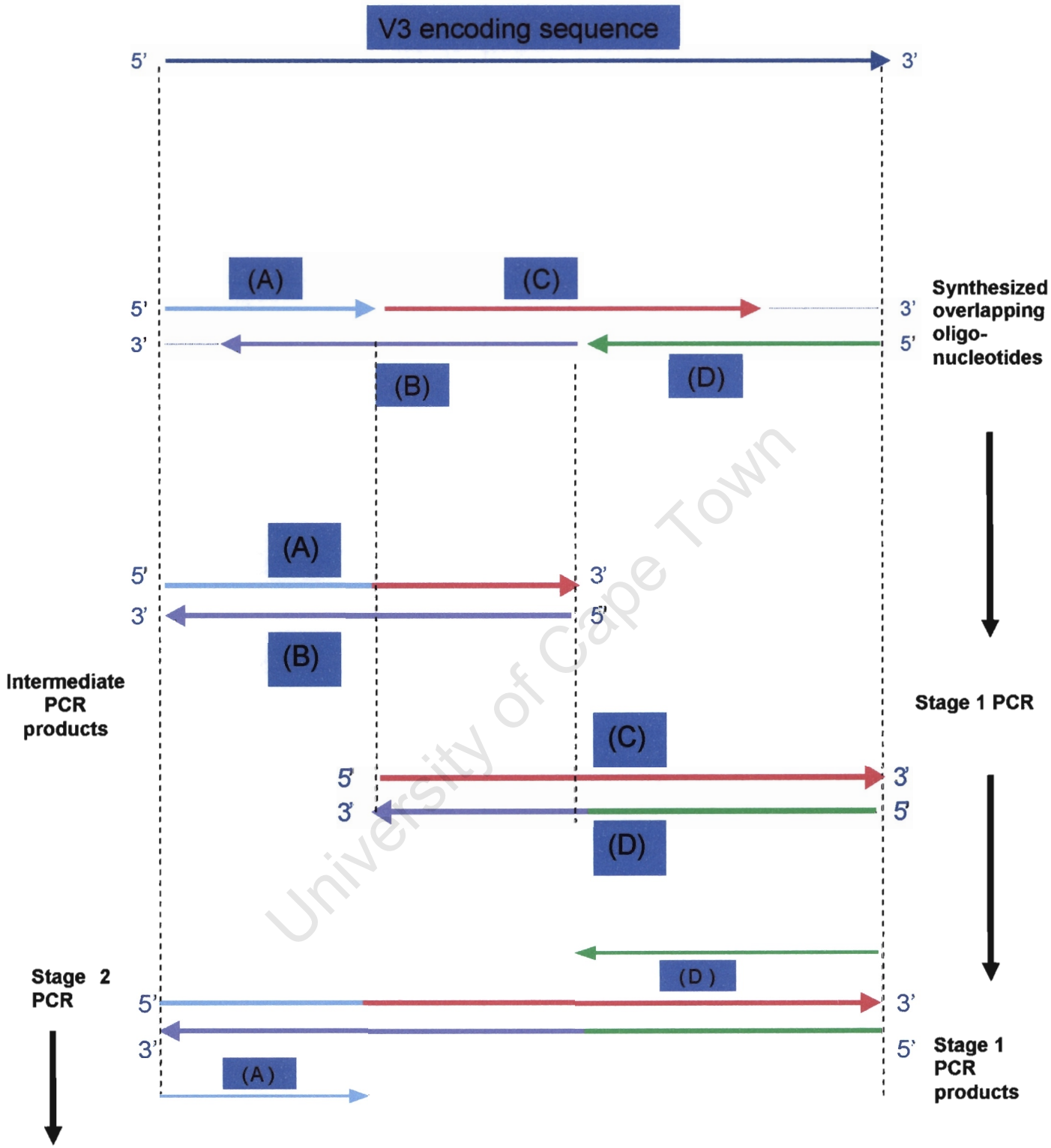


Figure 2.2 Diagrammatic representation of the PCR assembly process used for the assembly of the V3 loop encoding sequences.

2.2.2 General Cloning strategy used for inserting the epitope encoding DNA into sequencing and expression vectors

Assembly PCR products were initially ligated into the pGEM®-T Easy cloning/sequencing vector (Promega) (DNA insert ligations to either pGEM®-T Easy or pLSB vectors were routinely carried out in 10μl volumes using 1 unit of T4-DNA ligase (Roche). PCR product ligation into the pGEM®-T Easy vector was performed using reagents provided in the pGEM®-T Easy kit following manufacturers protocol in 30:1, insert : vector ratios.) and recombinant plasmids (pGEM+151a, 151b, 179a, 179b) were used to transform DH5α *E. coli* competent cells as described in Appendix A(VI). Plasmid DNA was isolated (Appendix A(II)) and the insert sequenced using an M13 forward primer (Appendix A(VII)) provided with the pGEM®-T Easy kit to verify the amplified sequence. DH5α *E. coli* transformants that were identified to contain the correct V3 epitope-encoding sequence were used for a large-scale plasmid DNA isolation (Appendix A(II)). Recombinant plasmid DNAs were digested in *NcoI* (Promega) restriction enzyme, Digests were carried out using 1 unit of enzyme/μg of DNA and incubated at 37°C for 2 hours before analysis. Supplier provided buffers and bovine serum albumin, which accompanied the specific enzyme, was used in each digest as per suppliers protocol. Digests were followed by column purification of digested DNA (Appendix A(V)). Column purified DNA was then digested in *NgoMIV* (New England Biolabs) restriction enzyme. The V3-encoding sequences were gel purified as described in Appendix A(V).

The V3-encoding fragments were ligated into the *NcoI* and *NgoMIV* restriction enzyme sites of pLSB 2295, 2109, 2296 vectors (Appendix B2, 4, 6) and insert

ligations to the pLSB vectors were carried out in 10:1 (insert:vector) ratios. Recombinant DNAs were used to transform competent *E. coli* DH5α cells (Appendix A(VI)) and plasmids were purified using a large-scale DNA isolation protocol (Appendix A(II)). The sequences of recombinant clones were verified by sequencing the pLSB2109 and pLSB2296 recombinants with the TMV CP forward primer (Appendix A(VII)), while the pLSB2295 recombinants were sequenced with a TMV CP reverse primer (Appendix A(VII)).

2.3 RESULTS AND DISCUSSION

2.3.1 Assembly of V3 epitopes (PCR assembly)

The V3 epitopes were successfully assembled using an assembly PCR technique. The expected approximately 75 bp band was identified in each assembly reaction (lanes 4, 5, 6 in Figure. 2.3).

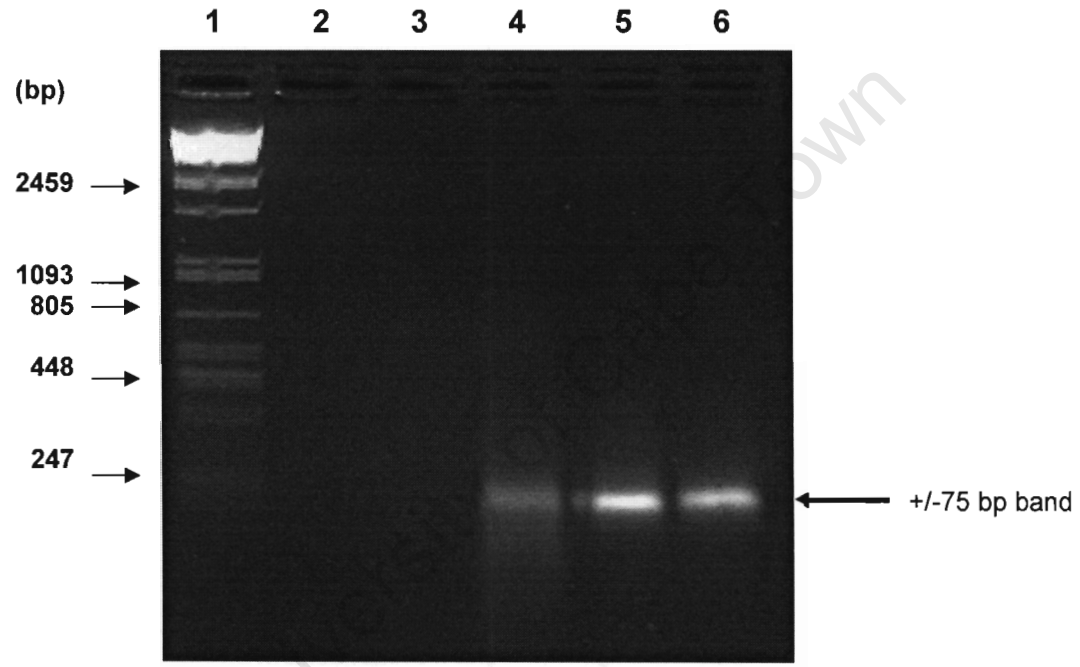


Figure 2.3 PCR assembly products resolved on a 2% agarose gel. Lane 1 contains a λPst DNA size marker, lanes 2 and 3 show the 0 DNA controls for stage 1 and 2 respectively and Lane 4 contains the Stage 1 experimental assembly PCR product, Lanes 5 and 6 show identical samples loaded in duplicate from the stage 2 V3 loop epitope assembly.

It is evident from duplicate lanes 5 and 6 (stage 2 PCR products) compared with lane (4) (stage 1 PCR product), that the stage 2 final product is a more defined product in terms of concentration and band specificity.

2.3.2 Cloning of Assembled V3 Epitope into Expression Vectors

The PCR product was screened to identify correctly assembled V3-encoding sequences by cloning the stage 2 PCR products into the pGEM T®-Easy cloning/sequencing vector (Appendix B1), and sequenced using the M13 forward primer. The *NcoI* and *NgoMIV* restriction enzyme digests of selected clones excised the insert from the pGem®-T Easy vector, which resulted in the expected approximately 75 bp band as well as an approximately 370 bp band as a result of an internal NgoMIV restriction site within the pGEM®-T Easy vector (Figure 2.4).

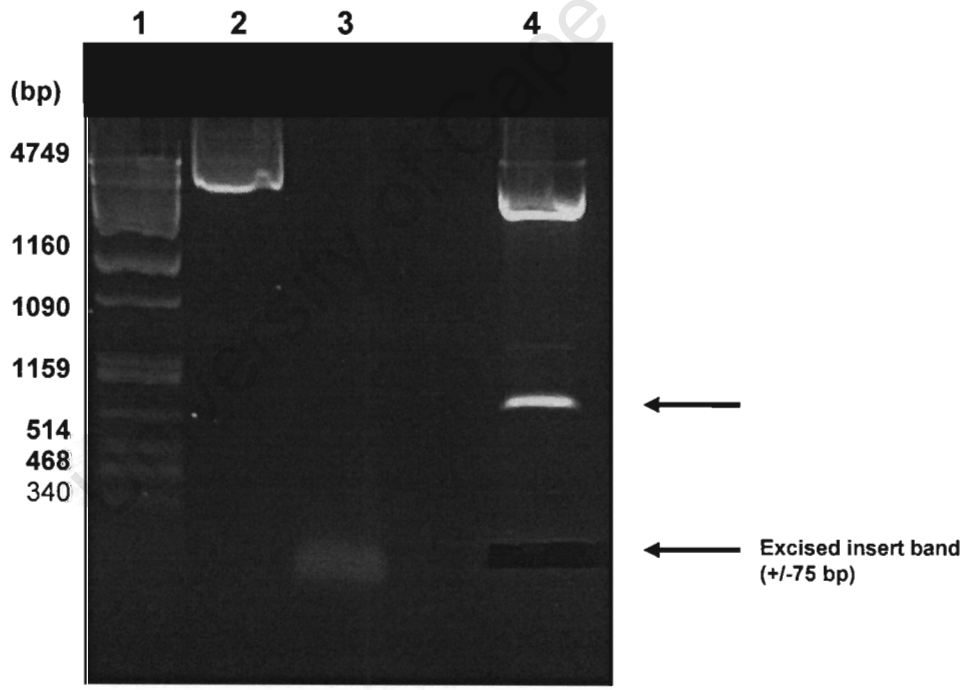


Figure 2.4 DNA products from an NcoI/NgoMIV double digest on a 1% agarose gel. Lane 1 contains the λPst DNA size marker, lane 2 contains the undigested pGem®-T Easy vector, lane 3 contains the stage 2 assembly PCR product as a size marker, lane 4 contains the NcoI/NgoMIV double-digested recombinant pGem®-T Easy vector from which the excised insert band was gel purified, indicated by the bottom arrow

As seen in Figure 2.4, the V3-epitope encoding bands were then cut out from the gel and purified from the gel slice.

V3 loop-encoding sequences were ligated to the *NcoI*- and *NgoMIV*- digested pLSB2295, 2109 and 2296 vectors (Appendix B2, 4, 6). The recombinant pLSB vectors were screened by sequencing using pLSB CP forward and reverse sequencing primers (Appendix A(VII)). Sequencing of recombinant clones showed that each of the pLSB2295, 2109, 2296 vectors contained each of the Du151a, 151b, 179a and 179b epitope-encoding sequences in the correct reading frame as indicated by the sequence translations that follow: (Figure 2.5 A-L)

(A)

pLSB2295 + Du151(a) (N-terminal fusion) translation

```
Total amino acid number: 181, MW=19889
Max ORF: 1-543, 181 AA, MW=19889

1       CCATGGGTAACAACAACACCCGGAAGAGCATCCGGATCGGACCAGGACAAACATTTTATGCA
1         M  G  N  N  N  T  R  K  S  I  R  I  G  P  G  Q  T  F  Y  A
61        ACCGCCGGCTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCATCAGCGTGG
21         T  A  G  S  Y  S  I  T  T  P  S  Q  F  V  F  L  S  S  A  W
121       GCCGACCCAATAGAGTTAATTAATTTATGTACTAATGCCTTAGGAAATCAGTTTCAAACA
41         A  D  P  I  E  L  I  N  L  C  T  N  A  L  G  N  Q  F  Q  T
181       CAACAAGCTCGAACTGTCGTTCAAAGACAATTCAGTGAGGTGTGGAAACCTTCACCACAA
61         Q  Q  A  R  T  V  V  Q  R  Q  F  S  E  V  W  K  P  S  P  Q
```

(B)

pLSB2295 + Du151(b) (N-terminal fusion) translation

```
Total amino acid number: 181, MW=19858
Max ORF: 1-543, 181 AA, MW=19858

1       CCATGGGTCGGAAGAGCATCCGAATAGGACCAGGACAGACATTCTATGCAACAGGAGAGATA
1         M  G  R  K  S  I  R  I  G  P  G  Q  T  F  Y  A  T  G  E  I
61        ATAGCCGGCTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCATCAGCGTGG
21         I  A  G  S  Y  S  I  T  T  P  S  Q  F  V  F  L  S  S  A  W
121       GCCGACCCAATAGAGTTAATTAATTTATGTACTAATGCCTTAGGAAATCAGTTTCAAACA
41         A  D  P  I  E  L  I  N  L  C  T  N  A  L  G  N  Q  F  Q  T
181       CAACAAGCTCGAACTGTCGTTCAAAGACAATTCAGTGAGGTGTGGAAACCTTCACCACAA
61         Q  Q  A  R  T  V  V  Q  R  Q  F  S  E  V  W  K  P  S  P  Q
```

(C)

2295 + Du179(a) (N-terminal fusion) translation

```
Total amino acid number: 180, MW=19731
Max ORF: 1-540, 180 AA, MW=19731

1       CCATGGGTGGCAACAACACCCGGAAGAGTATTCGAATTGGACCAGGACAGGCATTCTATACA
1         M  G  G  N  N  T  R  K  S  I  R  I  G  P  G  Q  A  F  Y  T
61      GCCGGCTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCATCAGCGTGGGCC
21       A  G  S  Y  S  I  T  T  P  S  Q  F  V  F  L  S  S  A  W  A
121     GACCCAATAGAGTTAATTAATTTATGTACTAATGCCTTAGGAAATCAGTTTCAAACACAA
41       D  P  I  E  L  I  N  L  C  T  N  A  L  G  N  Q  F  Q  T  Q
181     CAAGCTCGAACTGTCGTTCAAAGACAATTCAGTGAGGTGTGGAAACCTTCACCACAAGTA
61       Q  A  R  T  V  V  Q  R  Q  F  S  E  V  W  K  P  S  P  Q  V
```

(D)

pLSB2295 + Du179(b) (N-terminal fusion) translation

```
Total amino acid number: 180, MW=19822
Max ORF: 1-540, 180 AA, MW=19822

1       CCATGGGTCGGAAGAGTATTCGAATTGGACCAGGACAGGCATTCTATACAAACCACATCATC
1         M  G  R  K  S  I  R  I  G  P  G  Q  A  F  Y  T  N  H  I  I
61      GCCGGCTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCATCAGCGTGGGCC
21       A  G  S  Y  S  I  T  T  P  S  Q  F  V  F  L  S  S  A  W  A
121     GACCCAATAGAGTTAATTAATTTATGTACTAATGCCTTAGGAAATCAGTTTCAAACACAA
41       D  P  I  E  L  I  N  L  C  T  N  A  L  G  N  Q  F  Q  T  Q
181     CAAGCTCGAACTGTCGTTCAAAGACAATTCAGTGAGGTGTGGAAACCTTCACCACAAGTA
61       Q  A  R  T  V  V  Q  R  Q  F  S  E  V  W  K  P  S  P  Q  V
```

(E)

pLSB 2109 + Du151(a) (C-terminal fusion) translation

```
Total amino acid number: 182, MW=20034
Max ORF: 1-546, 182 AA, MW=20034

241     GTCACAGCACTGTTAGGTGCATTCGACACTAGAAATAGAATAATAGAAGTTGAAAATCAG
81       V  T  A  L  L  G  A  F  D  T  R  N  R  I  I  E  V  E  N  Q
301     GCGAACCCCACGACTGCCGAGACGTTAGATGCTACTCGTAGAGTAGACGACGCAACGGTG
101      A  N  P  T  T  A  E  T  L  D  A  T  R  R  V  D  D  A  T  V
361     GCCATAAGGAGCGCGATAAATAATTTAATAGTAGAATTGATCAGAGGAACCGGATCTTAT
121      A  I  R  S  A  I  N  N  L  I  V  E  L  I  R  G  T  G  S  Y
421     AATCGGAGCTCTTTCGAGAGCTCTTCTGGTTTGGTTTGGACCTCTGCCATGGGTAACAAC
141      N  R  S  S  F  E  S  S  S  G  L  V  W  T  S  A  M  G  N  N
481     AACACCCGGAAGAGCATCCGGATCGGACCAGGACAAACATTTTATGCAACCGCCGGCCCT
161      N  T  R  K  S  I  R  I  G  P  G  Q  T  F  Y  A  T  A  G  P
541     GCAACTTGA
181      A  T  *
```

(F)

pLSB 2109 + Du151(b) (C-terminal fusion) translation

```
Total amino acid number: 182, MW=20003
Max ORF: 1-546, 182 AA, MW=20003

241      GTCACAGCACTGTTAGGTGCATTCGACACTAGAAATAGAATAATAGAAGTTGAAAATCAG
81        V  T  A  L  L  G  A  F  D  T  R  N  R  I  I  E  V  E  N  Q
301      GCGAACCCCACGACTGCCGAGACGTTAGATGCTACTCGTAGAGTAGACGACGCAACGGTG
101       A  N  P  T  T  A  E  T  L  D  A  T  R  R  V  D  D  A  T  V
361      GCCATAAGGAGCGCGATAAATAATTTAATAGTAGAATTGATCAGAGGAACCGGATCTTAT
121       A  I  R  S  A  I  N  N  L  I  V  E  L  I  R  G  T  G  S  Y
421      AATCGGAGCTCTTTCGAGAGCTCTTCTGGTTTGGTTTGGACCTCTGCCATGGGTCGGAAG
141       N  R  S  S  F  E  S  S  S  G  L  V  W  T  S  A  M  G  R  K
481      AGCATCCGAATAGGACCAGGACAGACATTCTATGCAACAGGAGAGATAATAGCCGGCCCT
161       S  I  R  I  G  P  G  Q  T  F  Y  A  T  G  E  I  I  A  G  P
541      GCAACTTGA
181       A  T  *
```

(G)

pLSB2109 + Du179(a) (C-terminal fusion) translation

```
Total amino acid number: 181, MW=19876
Max ORF: 1-543, 181 AA, MW=19876

241      GTCACAGCACTGTTAGGTGCATTCGACACTAGAAATAGAATAATAGAAGTTGAAAATCAG
81        V  T  A  L  L  G  A  F  D  T  R  N  R  I  I  E  V  E  N  Q
301      GCGAACCCCACGACTGCCGAGACGTTAGATGCTACTCGTAGAGTAGACGACGCAACGGTG
101       A  N  P  T  T  A  E  T  L  D  A  T  R  R  V  D  D  A  T  V
361      GCCATAAGGAGCGCGATAAATAATTTAATAGTAGAATTGATCAGAGGAACCGGATCTTAT
121       A  I  R  S  A  I  N  N  L  I  V  E  L  I  R  G  T  G  S  Y
421      AATCGGAGCTCTTTCGAGAGCTCTTCTGGTTTGGTTTGGACCTCTGCCATGGGTGGCAAC
141       N  R  S  S  F  E  S  S  S  G  L  V  W  T  S  A  M  G  G  N
481      AACACCCGGAAGAGTATTCGAATTGGACCAGGACAGGCATTCTATACAGCCGGCCCTGCA
161       N  T  R  K  S  I  R  I  G  P  G  Q  A  F  Y  T  A  G  P  A
541      ACTTGA
181       T  *
```

(H)

pLSB2109 + Du179(b) (C-terminal fusion) translation

```
Total amino acid number: 181, MW=19967
Max ORF: 1-543, 181 AA, MW=19967

241      GTCACAGCACTGTTAGGTGCATTCGACACTAGAAATAGAATAATAGAAGTTGAAAATCAG
81        V  T  A  L  L  G  A  F  D  T  R  N  R  I  I  E  V  E  N  Q
301      GCGAACCCCACGACTGCCGAGACGTTAGATGCTACTCGTAGAGTAGACGACGCAACGGTG
101       A  N  P  T  T  A  E  T  L  D  A  T  R  R  V  D  D  A  T  V
361      GCCATAAGGAGCGCGATAAATAATTTAATAGTAGAATTGATCAGAGGAACCGGATCTTAT
121       A  I  R  S  A  I  N  N  L  I  V  E  L  I  R  G  T  G  S  Y
421      AATCGGAGCTCTTTCGAGAGCTCTTCTGGTTTGGTTTGGACCTCTGCCATGGGTCGGAAG
141       N  R  S  S  F  E  S  S  S  G  L  V  W  T  S  A  M  G  R  K
481      AGTATTCGAATTGGACCAGGACAGGCATTCTATACAAACCACATCATCGCCGGCCCTGCA
161       S  I  R  I  G  P  G  Q  A  F  Y  T  N  H  I  I  A  G  P  A
541      ACTTGA
181       T  *
```

(I)

pLSB2296 + Du151(a) (60S-Loop fusion) translation

```
Total amino acid number: 186, MW=20300
Max ORF: 1-558, 186 AA, MW=20300

121     GCTCGAACTGTCGTTCAAAGACAATTCAGTGAGGTGTGGAAACCTTCACCACAAGTAACT
41       A  R  T  V  V  Q  R  Q  F  S  E  V  W  K  P  S  P  Q  V  T
181     GTTAGGTTCCCTGGATCTCCCATGGGTAACAACAACACCCGGAAGAGCATCCGGATCGGA
61       V  R  F  P  G  S  P  M  G  N  N  N  T  R  K  S  I  R  I  G
241     CCAGGACAAACATTTTATGCAACCGCCGGCCCTTCTGGAGACTTTAAGGTATACAGGTAC
81       P  G  Q  T  F  Y  A  T  A  G  P  S  G  D  F  K  V  Y  R  Y
301     AATGCGGTATTAGACCCGCTAGTCACAGCACTGTTAGGTGCATTCGACACTAGAAATAGA
101      N  A  V  L  D  P  L  V  T  A  L  L  G  A  F  D  T  R  N  R
```

(J)

pLSB2296 + Du151(b) (60S-Loop fusion) translation

```
Total amino acid number: 186, MW=20269
Max ORF: 1-558, 186 AA, MW=20269

121     GCTCGAACTGTCGTTCAAAGACAATTCAGTGAGGTGTGGAAACCTTCACCACAAGTAACT
41       A  R  T  V  V  Q  R  Q  F  S  E  V  W  K  P  S  P  Q  V  T
181     GTTAGGTTCCCTGGATCTCCCATGGGTCGGAAGAGCATCCGAATAGGACCAGGACAGACA
61       V  R  F  P  G  S  P  M  G  R  K  S  I  R  I  G  P  G  Q  T
241     TTCTATGCAACAGGAGAGATAATAGCCGGCCCTTCTGGAGACTTTAAGGTATACAGGTAC
81       F  Y  A  T  G  E  I  I  A  G  P  S  G  D  F  K  V  Y  R  Y
301     AATGCGGTATTAGACCCGCTAGTCACAGCACTGTTAGGTGCATTCGACACTAGAAATAGA
101      N  A  V  L  D  P  L  V  T  A  L  L  G  A  F  D  T  R  N  R
```

(K)

pLSB2296 + Du179a (60S-Loop fusion) translation

```
Total amino acid number: 184, MW=20025
Max ORF: 1-552, 184 AA, MW=20025

121     GCTCGAACTGTCGTTCAAAGACAATTCAGTGAGGTGTGGAAACCTTCACCACAAGTAACT
41       A  R  T  V  V  Q  R  Q  F  S  E  V  W  K  P  S  P  Q  V  T
181     GTTAGGTCCCCTGGATCTCCCATGGGTGGCAACAACACCCGGAAGAGTATTCGAATTGGA
61       V  R  S  P  G  S  P  M  G  G  N  N  T  R  K  S  I  R  I  G
241     CCAGGACAGGCATTCTATACAGCCGGCCCTTCTGGAGACTTTAAGGTGTACAGGTACAAT
81       P  G  Q  A  F  Y  T  A  G  P  S  G  D  F  K  V  Y  R  Y  N
301     GCGGTATTAGACCCGCTAGTCACAGCACTGTTAGGTGCATTCGACACTAGAAATAGAATA
101      A  V  L  D  P  L  V  T  A  L  L  G  A  F  D  T  R  N  R  I
```

(L)

pLSB2296 + Du179b (60S-Loop fusion) translation

```
Total amino acid number: 185, MW=20233
Max ORF: 1-555, 185 AA, MW=20233

121     GCTCGAACTGTCGTTCAAAGACAATTCAGTGAGGTGTGGAAACCTTCACCACAAGTAACT
41       A  R  T  V  V  Q  R  Q  F  S  E  V  W  K  P  S  P  Q  V  T
181     GTTAGGTTCCCTGGATCTCCCATGGGTCGGAAGAGTATTCGAATTGGACCAGGACAGGCA
61       V  R  F  P  G  S  P  M  G  R  K  S  I  R  I  G  P  G  Q  A
241     TTCTATACAAACCACATCATCGCCGGCCCTTCTGGAGACTTTAAGGTATACAGGTACAAT
81       F  Y  T  N  H  I  I  A  G  P  S  G  D  F  K  V  Y  R  Y  N
301     GCGGTATTAGACCCGCTAGTCACAGCACTGTTAGGTGCATTCGACACTAGAAATAGAATA
101      A  V  L  D  P  L  V  T  A  L  L  G  A  F  D  T  R  N  R  I
```

Figure 2.5 A, B, C, D, E, F, G, H, I, J, K, L shows the relevant sequence translation data for the pLSB2295, pLSB2109 and pLSB2296 recombinant vectors containing the 151a, 151b, 179a and 179b V3 epitope inserts respectively.

CONCLUSION

Theoretical size estimations for TMV CPs resulting from the recombinant TMV-based vectors averaged about of 2 kDa larger than the wild-type CP as observed from the CP translations above. This suggested that the recombinant and wild-type TMV CPs could be distinguished by means of size variation.

As it was intended that only a segment (18/19 of 35/36 amino acids) of the V3 peptide be displayed on the TMV CP, it would probably not retain the native conformation and thus would be presented mostly as a linear epitope. Research has indicated that the binding of anti-V3 antibodies is most effective when the V3 domain retains its conformation (Schreiber *et al.,* 1997; Krachmarov *et al.,* 2001). As demonstrated in comparative studies, serum antibodies displayed much higher reactivity with a conformationally correct V3 fusion protein than with linear V3 peptides. The importance of these conformational epitopes was further demonstrated in a study conducted by Gorny *et al.* (2002) who showed that the strength of binding of anti-V3 antibodies to HIV virions correlates with neutralizing activity. The implications of this lie in the additional finding that the relative affinities of monoclonal antibodies were 1 to 2 orders of magnitude greater for the V3 region that retains its conformation than for the same epitope represented by a linear peptide. For this reason it may have been advisable to have inserted cysteine residues at positions flanking the epitope encoding sequence to enable the formation of the native hairpin structure through the formation of disulphide bonds.

Studies utilising CPMV expression vectors to present gp41 epitopes have been successful in raising neutralizing antibody responses (McLain *et al.*, 1995; 1996).

CPMV gp41 chimeric particle studies have also stressed the significance of presenting epitopes in their native conformations in order to stimulate specific neutralizing antibody responses (Burrati *et al.*, 1998). A similar study conducted using chimeric CPMV particles has also shown that the site of insertion of a foreign *Human Rhinovirus 14* (HRV-14) epitope in the coat protein can determine the overall 3-dimensional structural presentation of the foreign epitope (Taylor *et al.*, 2000). The structure assumed by the HRV epitope appeared to have altered its immunogenic properties as polyclonal antisera raised by the epitope assuming a closed loop conformation had a significantly enhanced ability to bind to intact HRV-14 particles compared with antisera raised against chimaeras presenting the same sequence as peptides with free C-termini. The antigen used to stimulate an immune response should preferably be capable of recognizing a broad range of different HIV-1 isolates. Gorny *et al.* (2002) have also shown that NAbs against conformational epitopes could neutralize a broad range of HIV-1 isolates, and it has been speculated that linear epitopes will be more type-specific in neutralizing activity.

Despite this, Joelson *et al.* (1997) have shown that a 13-amino acid sequence of V3 attached to the surface of a TBSV CP was detectable with the sera isolated from infected individuals, and also stimulated an immune response to the foreign HIV epitope when the chimaeric virus was injected into mice. Marusic *et al.* (2001) have also demonstrated that VLP's presenting the linear epitope "ELDKWA" from the transmembrane protein gp41 were capable of inducing the production of HIV-1 specific NAbs. The authors have speculated that the immunogenicity resulted from

the highly repetitive nature in which the epitope was displayed on the surface of the VLP.

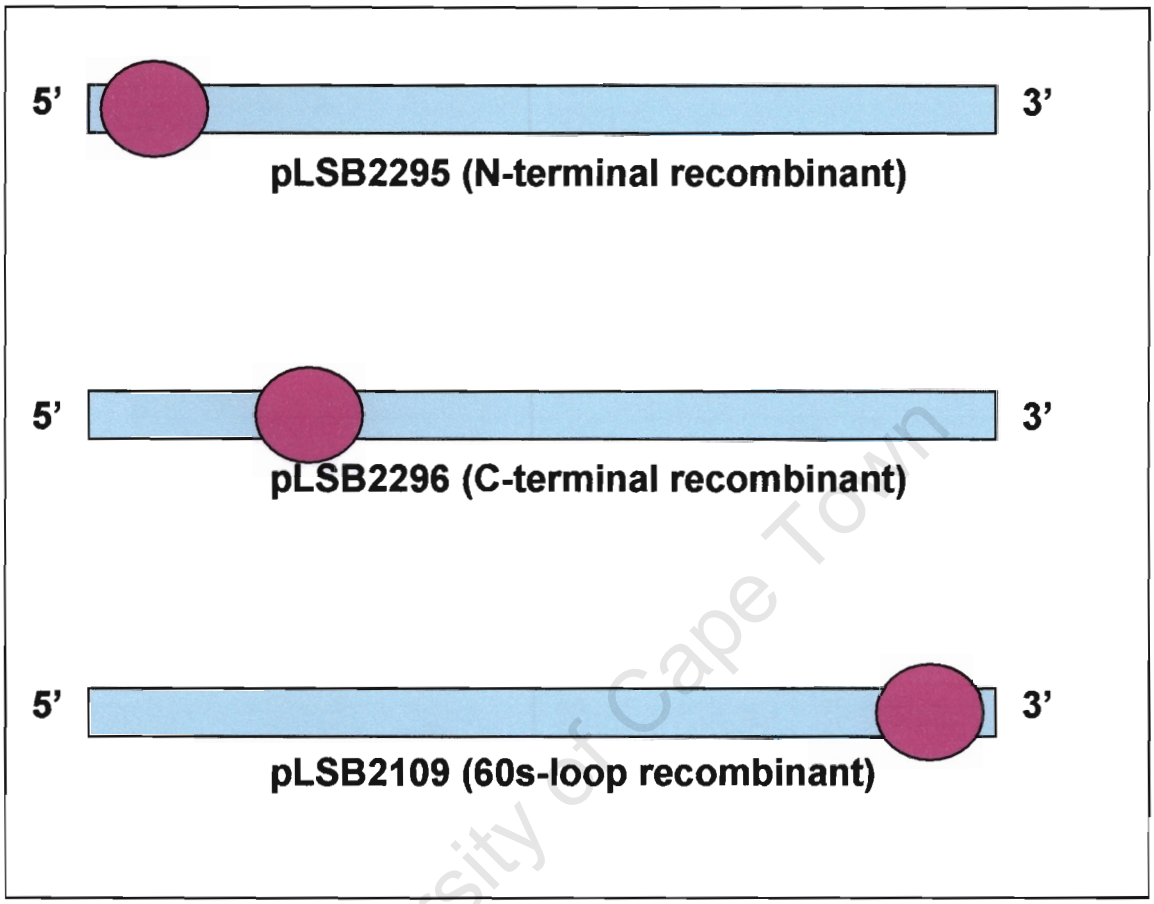


Figure 2.6 shows the relative positions of V3-epitope insertion illustrated by the pink dot in the TMV coat protein ORF illustrated by the green bars in the pLSB vectors.

In this part of the study the Du151 and Du179 HIV-1 subtype C V3 PND-encoding oligonucleotides were successfully assembled using an assembly PCR technique, each as 2 overlapping epitopes truncated at either the N- or C-terminus of the epitope. These resulted in epitope-encoding sequences Du151a, Du179a (C-terminal truncations), Du151b and Du179b (N-terminal truncations). These 4 epitope-encoding sequences were successfully cloned into the C-terminal, N-terminal and 60S loop region of the TMV CP in the pLSB2109, 2295 and 2296 TMV-based vectors respectively (Figure 2.6), resulting in a total of 12 constructs.

CHAPTER 3

Expression and Detection of Recombinant TMV in *Nicotiana benthamiana*

3.1 INTRODUCTION

TMV-based expression vectors have been successful in expressing foreign proteins in infected plants as either CP fusions or soluble protein, which have generally accumulated to high levels within the plant (Chapter 1). These high expression levels have in turn allowed for the development of economical methods to rapidly scale-up, extract and purify heterologously expressed proteins. The levels of the foreign protein or protein fusion produced vary and depend on several factors, which may include the protein itself, the vector expression system or the plant host (Culver, 2002). TMV has a broad host range and infects over 200 plant species including tobacco (*Nicotiana tabacum*), which is one of the highest biomass-producing crops in the world (Awram *et al.*, 2002). Tobacco thus serves as a good host for foreign protein expression. However, it has several problems as it contains numerous toxic alkaloids and phenolic compounds, which can complicate successful purification of any heterologously expressed protein (Awram *et al.*, 2002). For this reason the tobacco relative *N. benthamiana* has been recommended as a superior organic bioreactor as it does not contain the high levels of toxic phenolics and alkaloids associated with tobacco. Additionally, *N. benthamiana* allows the systemic spread of even attenuated TMV infection, enabling the production of large amounts of heterologously-expressed protein.

Infection entails the active replication of the recombinant virus and is manifested in distinct infection symptoms (leaf curling, mottling, chlorosis, necrosis, growth stunting). These are generally not too severe to kill the plant (Culver, 2002). In several cases disease symptoms within infected plants have been shown to be affected by the presence of the TMV CP and its intermediates (Culver, 2002). The

structure and charge of specific domains within the CP have been shown to modulate various infection symptoms and disease progression within infected plants (Banerjee *et al.,* 1995; Bendahmane *et al.,* 1999; Dawson *et al.,* 1988). In a specific example the disruption of certain CP domains by the addition of foreign epitopes has been shown to induce severe chlorosis and necrosis (Turpen *et al.,* 1995; Dawson *et al.,* 1988; Bendahmane *et al.,* 1999). Despite this the TMV CP has been shown to a certain degree to be redundant for the development of the typical mosaic symptoms in tobacco as mutant TMV not expressing CP still displayed mild mosaic symptoms in tobacco (Culver *et al.,* 1989). Infection symptoms therefore are not necessarily an indication of recombinant CP production and virion assembly.

In the vectors used in these experiments the TMV cDNA is carried on a pUC 18 plasmid backbone, which contains the T7 polymerase promoter sequence upstream of the TMV cDNA (Yanisch-Perron *et al*., 1985). This facilitates the amplification of the vector and allows generation of infectious RNA by *in vitro* transcription of the vector. As previously mentioned, one of the primary reasons that viral vectors are highly efficient is their ability to spread throughout the plant and express the foreign gene in all infected cells. A crucial step when attempting to establish a viral vector based protein expression system is to ascertain whether the viral vector in use is capable of efficiently surviving, replicating and spreading within the inoculated plant host. Success of viral vector infection though depends not only on how well the vector is adapted to survive in the plant but also on several factors with regard to how the inoculation was carried out. These factors may include the quality of the synthesised RNA transcript (i.e. degraded or not),

plant growth conditions, inoculation of a suitable leaf and the actual inoculation procedure.

In this chapter, the recombinant cDNA cloning vectors previously made are assessed for their ability to produce an infectious TMV genome, infect *N. benthamiana* plants and to establish a systemic infection. Several other questions are raised in this chapter, one of which is whether the TMV CP is able to retain the V3 insert or whether it reverts to a wild-type form. Both a V3 and TMV-specific antibody are used to distinguish wild-type and recombinant CPs. As the primary aim of this study was to create a multivalent V3 display particle, virion assembly is important: thus, I investigated whether the presence of the V3 epitope sterically hinders virion assembly of the various N-, C-terminal and 60S-loop fusions, using electron microscopy.

3.2 MATERIALS AND METHODS

3.2.1 *In vitro* transcription

Capped transcripts from full-length recombinant TMV cDNA clones were generated using the Ribomax^{TM} Large Scale RNA Production System – T7 kit (Promega). Transcription reactions were carried out according to the manufacturer's instructions using 5μg of template DNA per 50μl reaction. Prior to the transcription reaction, DNA templates were treated with 1u/μg DNA of human placental RNase inhibitor (Roche) for 1 hour at 37° C. After synthesis RNA samples were stored at -70° C. Results of the *in vitro* transcription were determined by electrophoresis on a 1% TBE agarose gel (Appendix A(V)). Two microlitres of transcribed RNA was loaded onto the gel and a total of 300ng of untranscribed plasmid DNA was also loaded as a control.

3.2.2 Inoculation of plants and growth conditions

Nicotiana benthamiana seeds (LSBC 2002, K.E. Palmer, LSBC, CA, USA) were sown individually in seedling trays and covered with plastic until they had germinated. Seedlings were then transplanted after 3 weeks into pots. By 5 to 6 weeks the plants were ready for inoculation. Plants were grown at 45% humidity, a constant temperature of 28° C and subjected to 16 hours of light and 8 hours of dark. Lighting was supplied by 4 high-output fluorescent lamps (Phillips L58W/205) suspended 50cm above the plants. Plants were inoculated by means of mechanical abrasion of the leaf surface. Each inoculation used 7.5μl of each 50μl *in vitro* transcription reaction with an RNase free abrasive powder (Celite), which was gently rubbed onto one of the upper leaves in such a way that the leaf was not

visibly abraded. Each TMV construct was inoculated onto a set of 6 plants which were monitored daily for the presence of infection symptoms.

Systemic spread was usually visible 10 days post infection (dpi), at which point 25-35g of leaf material was harvested from each set of plants and used for TMV extraction.

3.2.3 TMV extraction protocols

TMV was purified from infected plants 10 dpi as described elsewhere (Chapman, 1998).

3.2.3.1 Wild-type TMV

Harvested leaf material (25-35g) was homogenised in liquid nitrogen and resuspended in 60ml 50mM phosphate buffer, pH8 containing 1% 2-mercaptoethanol (v/v). Chlorophyll and cellular debris were coagulated by the addition of butan-1-ol (0.8ml/10ml homogenate). Viral particles were precipitated using polyethylene glycol (4% w/v) and concentrated by centrifugation. The viral pellet was dissolved in 10mM pH8 phosphate buffer. Virus yield was determined by measuring the absorbance at 260nm and assuming an extinction coefficient of 3.

3.2.3.2 Recombinant TMV

It was evident that the addition of the foreign V3 epitopes to the surface of TMV had altered its physiochemical properties. Specifically, the excess positive charge conferred by the V3 epitopes apparently made the recombinant CP/virions insoluble at pH8. Harvested leaf material (25-35g) was therefore homogenised in

liquid nitrogen and resuspended in 60ml 50mM phosphate buffer, pH5 containing 1% 2-mercaptoethanol (v/v). The remainder of the protocol was carried and as in 3.2.3(a) except that all phosphate buffers were used at pH5.

3.2.4 Western blotting

The procedure followed the methods set out in Appendix A(VIII) and A(IX), describing SDS-PAGE and immunoblotting. Blots were initially probed with a 1/5000 dilution of rabbit anti-TMV CP specific polyclonal serum.

3.2.5 Enzyme-linked immunosorbent assay (ELISA)

ELISAs were routinely carried out as described in Appendix A(X) for the detection of the V3-epitope peptide on the surface of recombinant TMVs. For this assay a V3 peptide with the sequence N-(CTRPNNNTRKSIRIQRGPGRAFVTIGK IGNMRQAHCN)-C as well as a mouse-derived monoclonal antibody specific for the N-(KSIRI)-C sequence within each of the 151a, 151b, 179a and 179b epitopes was used at a 1/2000 dilution to establish a V3 peptide-specific standard curve. Both antibody and V3 peptide were acquired from the National Institute for Biological Standards and Control (UK; repository reference numbers EVA331 and ARP792.3 respectively). Detection of V3 peptide on the surface of recombinant TMV CPs required that the wells in the ELISA plate be coated with 10µg of each TMV CP recombinant and initially probed with a 1/2000 dilution of the above mouse V3-specific monoclonal antibody. This was followed by probing wells with a 1/10000 dilution of alkaline phosphatase conjugated goat anti-mouse secondary antibody (Sigma-Aldrich).

3.2.6 Electron microscopy

3.2.6.1 Sample grid preparation

Sample grids were analysed using the LEO 912 OMEGA Energy Filter Transmission Electron Microscope. Carbon coated copper grids were used for the visualisation of TMV extracts. Grids were applied carbon coated side down onto a 15µl volume of undiluted TMV extract for 60 minutes. Grids were then washed twice by applying them on 15µl volumes of distilled water for 30 seconds per wash. Excess moisture was blotted from the grid between each wash. Grids were next stained with 2% uranyl acetate for 2 minutes, before excess stain was blotted from the grid. Grids were allowed to dry and stored in a grid holder.

3.2.6.2 Antibody decoration

Grids were applied carbon coated side down onto a 20µl volume of undiluted TMV extract for 60 minutes, then washed twice by applying them on 20µl volumes of phosphate buffered saline (PBS) for 1 minute per wash. Excess moisture was blotted from the grid between each wash. Grids were next blocked by applying them onto 20µl volumes of 1% bovine serum albumin (BSA) in PBS for 30 minutes, and then washed as before. Grids were then probed with 1/500 dilution of either the anti-TMV or anti-V3 antibodies by applying grids onto the appropriate antibody dilution for 60 minutes, and next washed five times for 2 minutes each in distilled water as before. Grids were next stained with 2% uranyl acetate for 2 minutes, before excess stain was blotted from the grid. They were stored in a grid holder.

3.3 RESULTS

3.3.1 *In vitro* transcription and inoculation of plants

In vitro transcription reactions were successful in synthesizing the TMV RNA as indicated by the broad bands in Lanes 3, 5 and 7 in Figure 3.1.

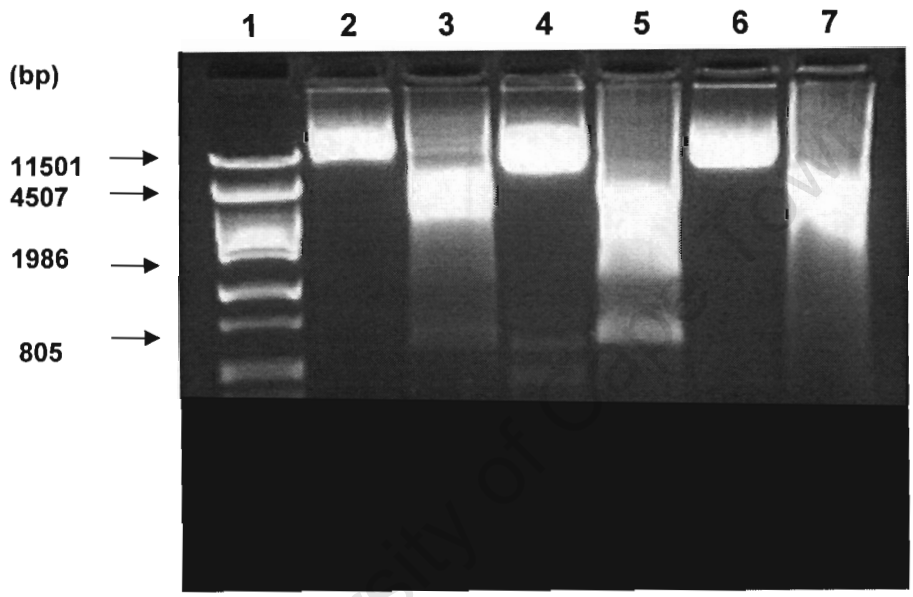
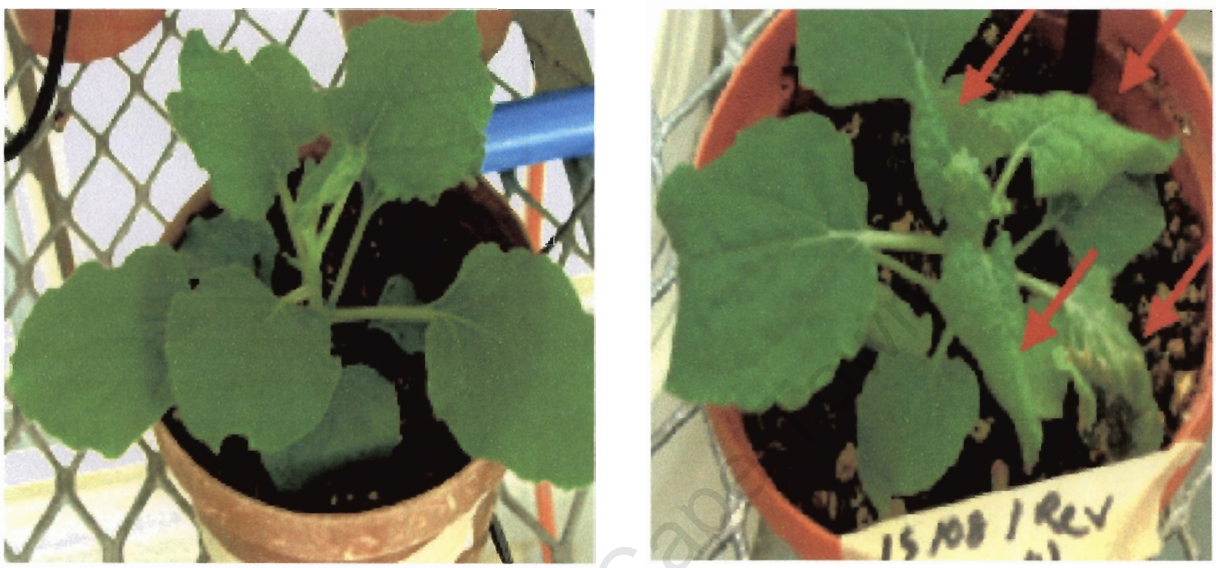


Figure 3.1 *In vitro* transcription reaction products are shown in Lanes 3, 5 and 7 synthesized from their corresponding recombinant TMV based vector template DNA shown in Lanes 2, 4 and 6 respectively. Lane 1 contains the λPst DNA size marker.

The *in vitro* synthesized RNAs were used for the inoculation of *N. benthamiana* plants using a leaf abrasion technique explained in 3.2.2. Each day post inoculation (dpi), *N. benthamiana* plants were observed for the development of the classic TMV infection symptoms, which included leaf curling, mottling, chlorosis, necrosis and/or growth stunting which is clearly distinct in comparison to a healthy, uninfected plant (Figures 3.2(a) and (b)). In general, infection symptoms for all 12

constructs initially manifested at 3-4 dpi in the uppermost leaves of the plant and subsequently spread to lower leaves, establishing a systemic symptomatic infection at 10 dpi.



(a) (b)

Figure 3.2 (a) A healthy uninfected *N. benthamiana* plant displaying no TMV infection symptoms. (b) A *N. benthamiana* plant that has been infected with one of the recombinant TMV constructs and displays leaf curling, mottling, chlorosis and a general stunting of growth indicated by the red arrows.

Leaves were harvested from the inoculated plants at 10 dpi to ensure that an optimal yield in both leaf material and TMV particles was obtained. A longer infection period resulted in the rapid loss of exploitable leaf tissue as a result of severe systemic necrosis.

3.3.2 TMV CP detection

TMV CP was extracted from the harvested infected leaf material by means of optimised extraction protocols for either the wild-type CP (3.2.3.1) or the recombinant CP (3.2.3.2), (see discussion 3.4). Extraction of TMV CP from

infected leaf material was confirmed using western blotting (Figures 3.3.1, 3.3.2, and 3.3.3).

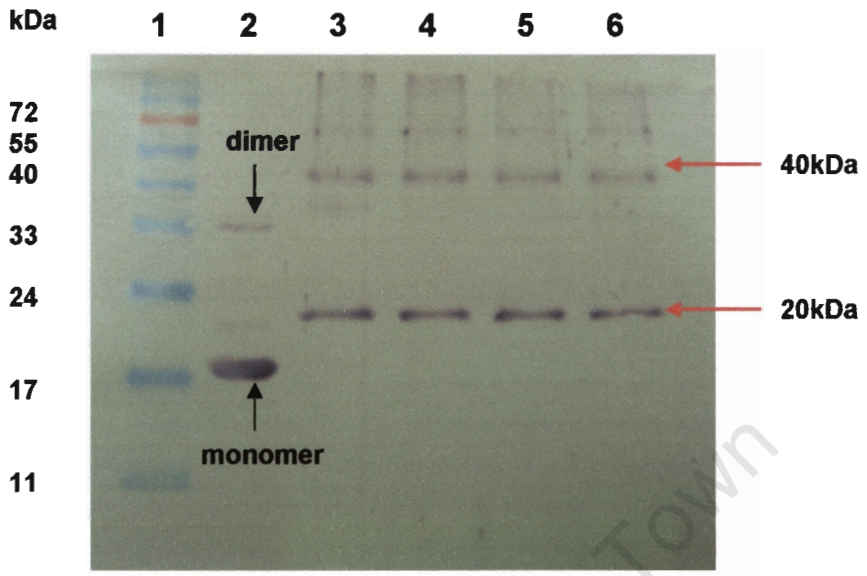


Figure 3.3.1 Western blot of pLSB2296 TMV recombinants. Lane 1 molecular weight marker, Lane 2 pLSB2296 non-recombinant extract, Lane 3 pLSB2296+151a recombinant extract, Lane 4 pLSB2296+151b recombinant extract, Lane 5 pLSB2296+179a recombinant extract, Lane 6 pLSB2296+179b recombinant extract. Black arrows indicate the position of wild-type TMV CP monomers (+/-18kDa) and dimers (+/-36kDa), while the red arrows indicate the position of larger recombinant TMV CP monomers (+/-20kDa) and dimers (+/-40kDa).

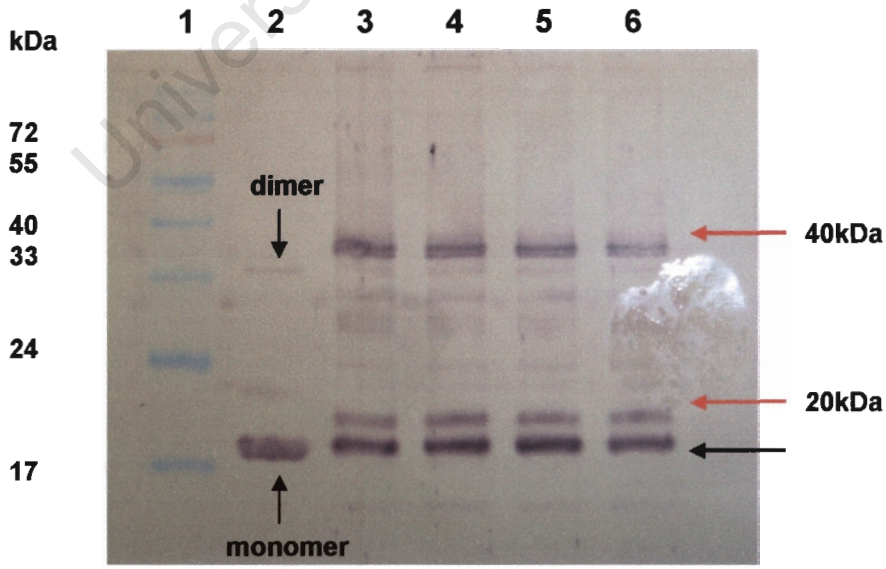


Figure 3.3.2 Western blot of pLSB2109 TMV recombinants. Lane 1 molecular weight marker, Lane 2 pLSB2109 non-recombinant extract, Lane 3 pLSB2109+151a recombinant extract, Lane 4 pLSB2109+151b recombinant extract, Lane 5 pLSB2109+179a recombinant extract, Lane 6 pLSB2109+179b recombinant extract. Black arrows indicate the position of wild-type TMV CP monomers (+/-18kDa) and dimers (+/-36kDa), while the red arrows indicate the position of larger recombinant TMV CP monomers (+/-20kDa) and dimers (+/-40kDa).

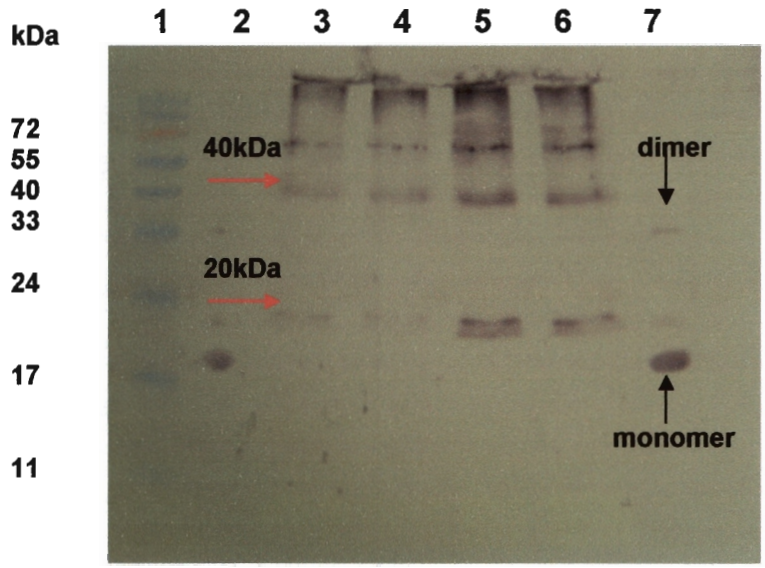


Figure 3.3.3 Western blot of pLSB2295 TMV recombinants. Lane 1 molecular weight marker, Lane 2 and 7 pLSB2295 non-recombinant extract, Lane 3 pLSB2295+151a recombinant extract, Lane 4 pLSB2295+151b recombinant extract, Lane 5 pLSB2295+179a recombinant extract, Lane 6 pLSB2295+179b recombinant extract. Black arrows indicate the position of wild-type TMV CP monomers (+/-18kDa) and dimers (+/-36kDa), while the red arrows indicate the position of larger recombinant TMV CP monomers (+/-20kDa) and dimers (+/-40kDa).

From the western blots (Figures 3.3.1-3) it is evident that the systemic spread of infection within the plants was accompanied by the expression and synthesis of recombinant TMV CP. It is evident that varying levels of expression occurred between the between the different constructs. The pLSB2296 recombinants (Figure 3.3.1) appear to exhibit the best results, as the recombinant CPs appeared as distinct 20 (monomer) and 40kDa (dimer) bands on the blot. These are indicated by the red arrows easily distinguished from the smaller wild-type bands indicated by the black arrows. None of the pLSB2296 recombinants (60S-loop) appeared to have reverted to a wild-type form, which indicates pLSB2296 construct stability. The pLSB2109 recombinants (C-terminal, Figure 3.3.2) on the other hand, did not appear as stable; there was a distinct wild-type 18kDa monomer band (black arrow) instead of only the 20kDa (monomer) and 40kDa

(dimer) bands (red arrow) present in all pLSB2109 recombinant extracts. The pLSB2295 recombinants continually proved difficult to extract in significant quantities compared to the pLSB2109 and pLSB2296 recombinants. As seen on the blot in figure 3.3.3, recombinant bands were barely visible in comparison to the other 2 blots in which identical extraction volumes were loaded. It was also noted from this blot that wild-type CP bands are either not present or occur at much lower concentrations compared to the recombinant bands indicated by the red arrows, which may suggest that the N-terminal fusion in this vector interfered with TMV CP expression and drastically reduced expression levels.

3.3.3 V3 Detection

As a mere size difference between the wild-type and recombinant CPs was not sufficient to verify the presence of the V3 peptide on the surface of the recombinant TMV CPs, an immunoassay was employed to prove this. As identified in chapter 2 (2.3), the foreign peptide comprises approximately only 10% of the recombinant CP and the mouse antibody used to detect the V3 peptide is targeted towards a highly specific 5 amino acid sequence (N-KSIRI-C) that occurs within all variants of the peptide used. For this reason, high concentrations of recombinant TMV CPs were required for the detection of the V3 peptide. An ELISA was therefore employed instead for the detection of the V3 peptide on the TMV CP surface. To allow detection of the V3 peptide, 10µg amounts of each of the 12 TMV recombinants were used to coat the ELISA plates, which were then probed with the above monoclonal V3 loop specific antibody.

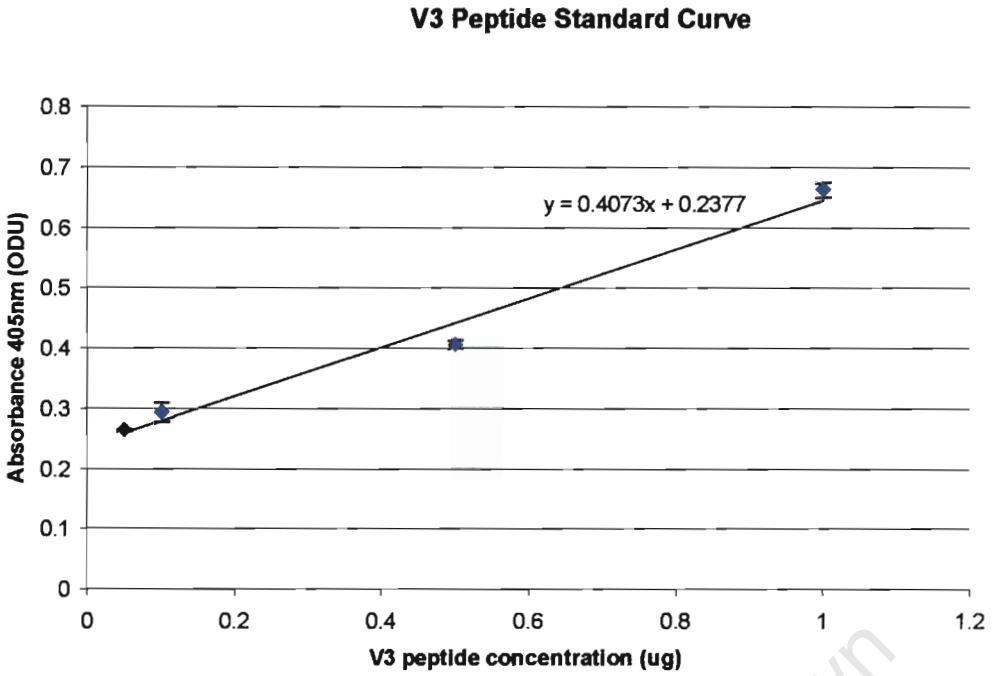


Figure 3.4 Standard curve for V3 peptide that shows homology to the V3 peptides displayed by the various constructs. Displayed on the graph is the formula for estimating the amount of V3 peptide between the ranges of 100ng to 1µg.

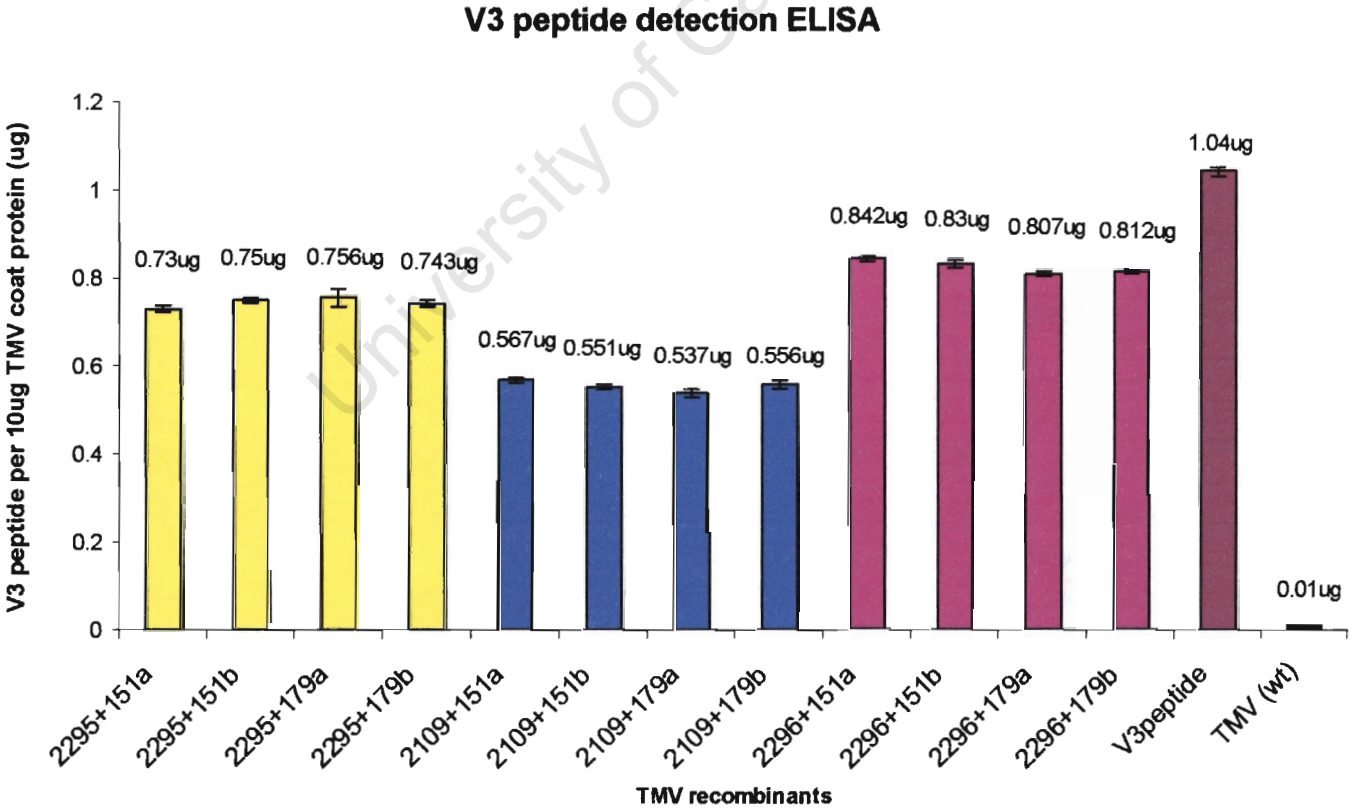


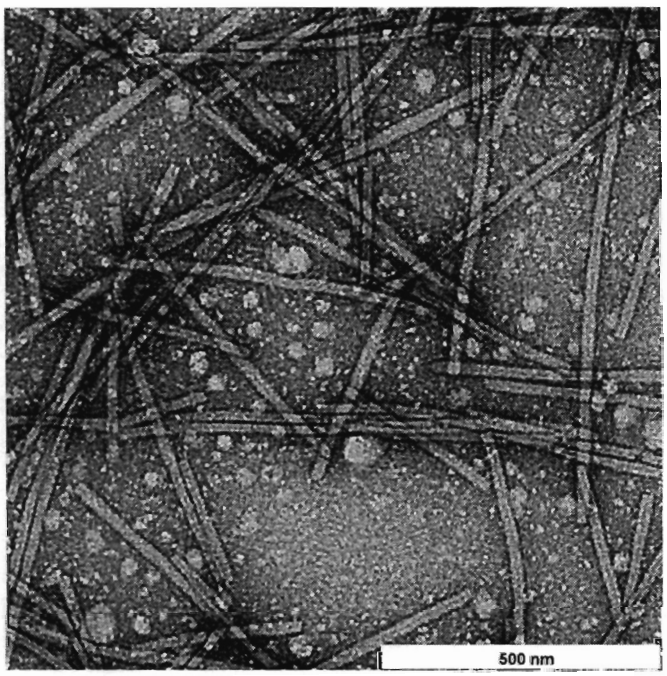
Figure 3.5 V3 detection ELISA results for the recombinant TMV vectors. Results of pLSB2295 recombinant vectors are displayed in yellow, results of pLSB2109 recombinant vectors are displayed in blue, results of pLSB2296 recombinant vectors are displayed in pink and positive (1ug V3 peptide) and negative (10ug wild-type TMV) control results are displayed in purple. All V3 peptide levels that occur per 10ug of recombinant TMV CP is indicated above the respective bar.

The formula generated by the V3 peptide standard curve (y = 4.073x – 0.2377) in figure 3.4 was used to calculate the levels of V3 peptide present in 10µg of each recombinant CP from ELISA results (Figure 3.5). Results from the ELISA indicated that the V3 peptide is expressed by the recombinant virus, although at varying levels. As the foreign peptide occupied approximately 10% of the total recombinant CP, the V3 peptide levels were not expected to exceed 1µg/10µg recombinant TMV CP. The pLSB2295, pLSB2109 and pLSB2296 recombinant CPs on average accumulated to approximately 75% (0.75µg), 55% (0.55µg) and 82% (0.82µg) of this maximum value respectively. These varying levels of expression are found to correlate well with the western blot results (Figures 3.3.1, 2 and 3). As noted in the pLSB2295 and pLSB2296 western blots (Figure 3.3.1 and 3.3.3) only the larger recombinant CP monomer and dimer bands were distinctly visible, indirectly inferring a higher V3 peptide level compared to the pLSB2109 vector recombinants. In contrast the pLSB2109 western blot displayed a significant level of monomeric wild-type revertant CPs that has presumably lost the V3 peptide encoding sequence, which also correlates well with the low (+\-55%) levels of V3 peptide generated by the pLSB2109 vector recombinants. The presence of the V3 peptide on the surface of all recombinant TMVs was thus confirmed.

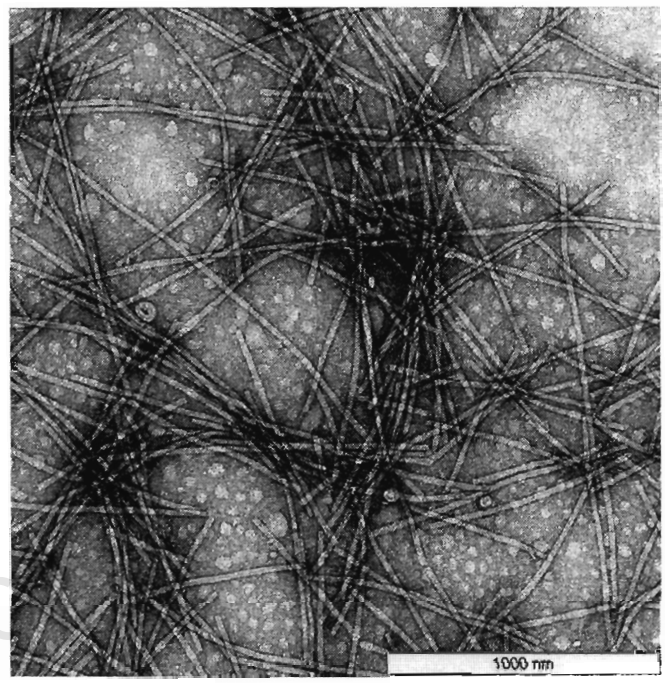
3.3.4 Virion Assembly

As a primary aim of this project was to produce a multivalent peptide presenting system, I investigated whether the recombinant CPs were capable of assembling into the highly repetitive, peptide-presenting virion structures. Recombinant TMV extracts were visualised by means of transmission electron microscopy and all constructs were found to be proficient at producing protein that assembled into the

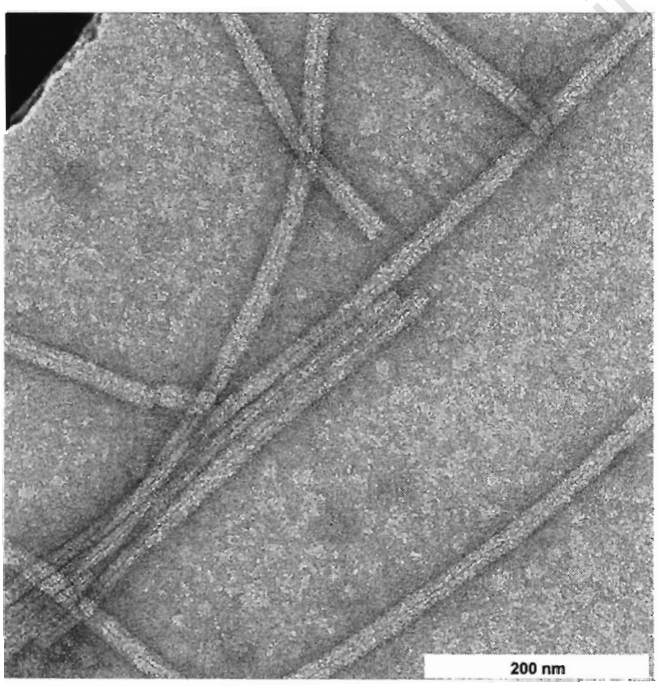
polymeric rod like structures typical of TMV. Figure 3.6 (b), (c) and (d) illustrate the typical structures observed for each set of TMV recombinants, which appear identical to the non-recombinants (Figure 3.6 (a)).



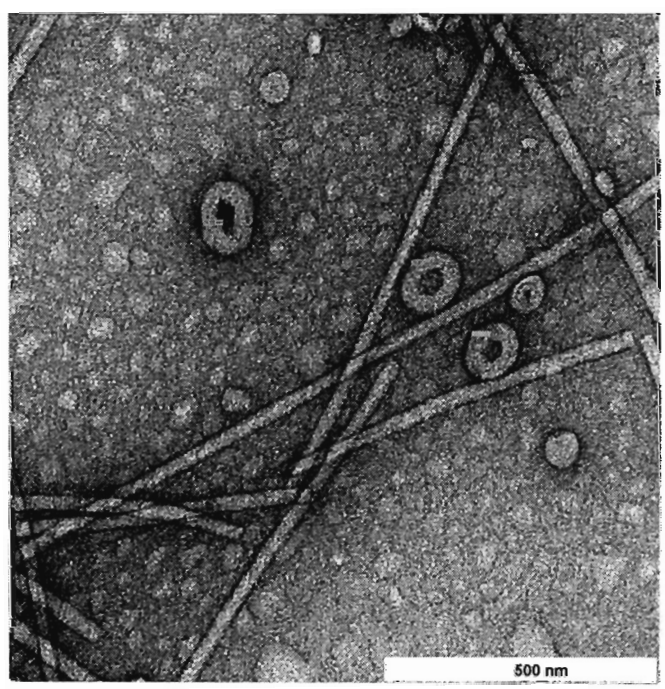
(a)



(b)



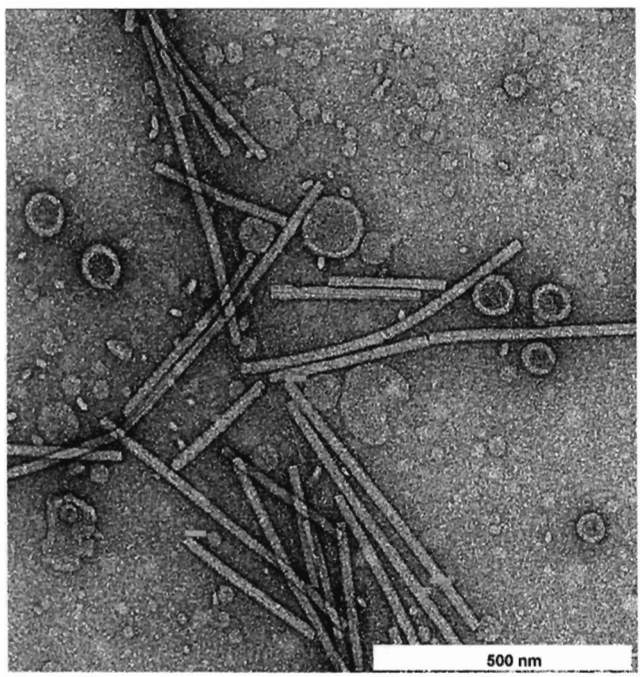
(c)



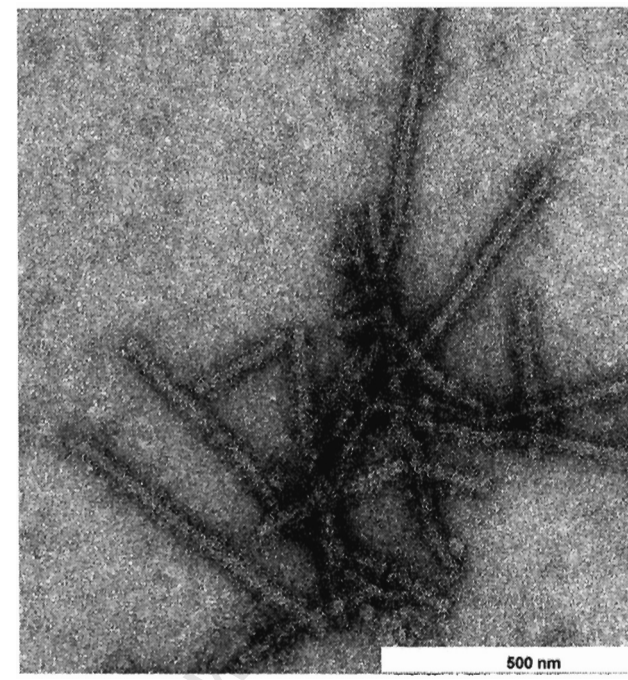
(d)

Figure 3.6 Transmission electron micrographs of TMV extracts displaying assembled virions. (a) pLSB2109 non-recombinant, (b) pLSB2109+151a recombinant, (c) pLSB2296+151a recombinant, (d) pLSB2295+151a recombinant.

As a means of visually confirming the presence of the V3 peptide on the surface of the assembled recombinant virions, the pLSB2109+151a extract was subjected to a V3 peptide-specific antibody decoration technique. If the V3 peptide is present on the surface of the assembled virion it would appear blurred by the presence of the V3 peptide-specific antibodies. The results shown in figures 3.7 (a), (b) and (c) verify the presence of the V3 peptide on the surface of the assembled pLSB2109+151a recombinant, as it appears with a shaded haze around the virion structures (Figure 3.7 (b)), while the pLSB2109 non-recombinants clearly do not bind any of the V3 peptide-specific antibody (Figure 3.7 (a)). In addition, the pLSB2109 non-recombinant vector is bound by the anti-TMV polyclonal serum (Figure 3.7 (c)). This finally confirmed that the virions do assemble and display V3 peptide epitopes on their surface.

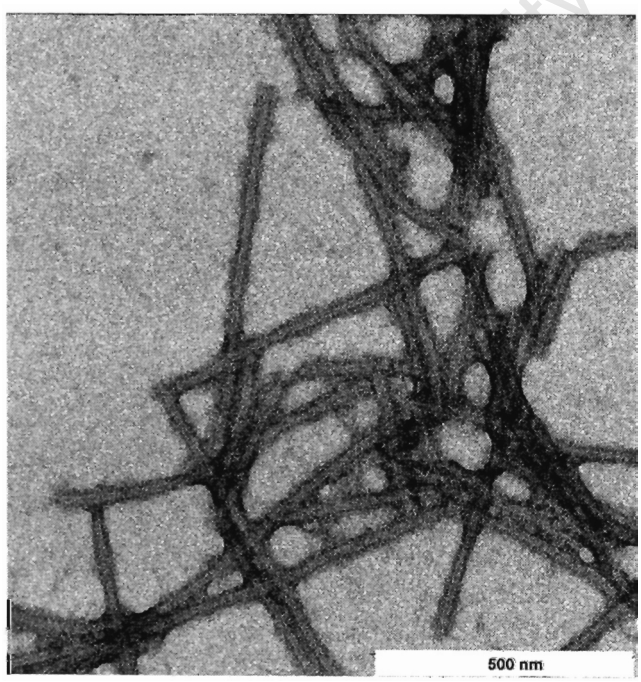


(a)



(b)

Figure 3.7 Transmission electron micrographs of V3 peptide and TMV specific antibody decorated TMV particles. (a) pLSB2109 non-recombinant extract probed with the V3 peptide-specific antibody, (b) pLSB2109+151a recombinant probed with the V3 peptide-specific antibody, (c) pLSB2109 non-recombinant probed with a TMV CP specific polyclonal serum.



(c)

3.4 CONCLUSION

Several important points have been made in this chapter. The 3 types of recombinant vectors used were all shown to be proficient at initially infecting the inoculated plant and establishing a systemic infection, without excessively damaging the infected plant. This in turn has aided the extraction yield. Once plants were infected, the recombinant viral genomes expressed the recombinant viral CPs as seen in the western blots (Figures 3.3.1-3). The levels of expression and hence the recombinant CP yield from inoculated plants however, did vary depending on the vector used. The pLSB2295 (N-terminal fusion) vector recombinants gave the lowest CP yield. This observation is not completely unexpected, as it was shown that the TMV CP promoter extends into the CP ORF and thus the insertion of a foreign sequence at the 5' end of the CP ORF may interfere with expression by disrupting the promoter (Grdzelishvili *et al.,* 2000). Furthermore, sequence deletion analysis by Grdzelishvili *et al.* (2000) has demonstrated that the core (minimal) promoter sequence lies between nucleotide-69 and +12 from the CP open reading frame start position as opposed to the full promoter, which lies between -157 and +54 and which would allow maximal gene expression. Thus the low expression levels exhibited by the N-terminal CP fusions appears to be due to disruption of the CP promoter, which had significant effect on recombinant CP expression levels. Both the pLSB2296 (60S-loop) and pLSB2109 (C-terminal) recombinants expressed higher levels of CP within the infected plants, which is also not unexpected as C-terminal CP fusions are most frequently used, and both fusions do not interfere with the promoter sequence (Fitchen *et al.,* 1995; Turpen *et al.,* 1995; Yusibov *et al.,* 1997; Koo *et al.,* 1999).

Wild-type CP revertants were observed for the pLSB2109 CP recombinants as demonstrated both in western blots and more accurately in the ELISA test, which showed lower (+/-55%) than expected V3 peptide levels contained in defined amounts of recombinant CP. This would render the pLSB2109 vector recombinants suboptimal choices for candidate vaccines as a result of the vector instability, when compared to the pLSB2296 (60S-loop) vector recombinants, which displayed an almost optimal level of recombinant CP and V3 peptide levels (+/-82%).

Electron micrographs conclusively showed that the recombinant TMV CPs are capable of assembling into polyepitope-presenting virions, and that the foreign epitopes do not appear to significantly sterically hinder virion assembly. The addition of the foreign epitope sequences to the TMV CP appears, however, to also have altered the physiochemical properties of the CP. Attention was first drawn to this fact by the observation that recombinant CPs were insoluble at the physiological pH7.4. This was theorised to have arisen due to the highly positive nature of the V3 peptides, which changed the overall charge of the TMV CP and required that the TMV extraction protocol be modified: I used buffers at pH5 to promote recombinant CP solubility. Similar insolubility problems have previously also been documented with recombinant TMV CP fusions in studies conducted by Fitchen *et al.,* (1995) and Koo *et al.,* (1999). The alteration in virion charge has previously also found to be associated with an increase in virion length up to 3 or 4 times that of a wild-type virion (300nm) (Lu *et al.,* 1996). This could account for the above electron micrographs of recombinant virions that display a corresponding array of virion lengths.

This chapter has demonstrated that TMV-based vectors have been synthesized that are capable of displaying multiple copies of the V3 peptide. They are thus suitable for testing in an animal model system to determine their immunogenicity and eventually their ability to possibly facilitate neutralization of HIV infection.

CHAPTER 4

Immunogenicity of Recombinant TMV Particles

4.1 INTRODUCTION

Immunogenicity analysis of proposed vaccine candidates usually requires the use of one or more animal models. Generally, animal models are selected based on the purpose of the proposed vaccine, although several other physical factors also play key roles in helping to select an animal model for immunogenicity analysis. These factors may include the required animal cohort size, space, time and financial restrictions; thus, the selection of an animal model requires significant forethought. Furthermore, the selected animal model has direct bearing on the possible methods (route) of vaccine administration and hence the desired type of immune response.

Typically, vaccine immunogenicity analysis has utilized either primate (baboon, macaque, chimpanzee) or small animal (mouse, rabbit, rat, guinea pig) animal model systems (Letvin *et al.*, 2001; Marusic *et al.*, 2001; Staczek *et al.*, 2000, Liao *et al.*, 2000 and 2004; Oscherwitz *et al.*, 1999). Small animal models are comparatively more simplistic as they occupy less space, require smaller inoculum doses and are overall easier and more cost-effective to maintain. Primate models on the other hand, present a system that more closely resembles that for which the vaccine is intended, when developing a vaccine for human purposes. It is therefore assumed that primate animal models would be the better option when testing a proposed HIV vaccine. Despite this, several proposed HIV vaccines have generated promising immune responses within small animal model systems (Marusic *et al.*, 2001; Liao *et al.*, 2000 and 2004; Yusibov *et al.*, 1997, Durrani *et al.*, 1998).

The "newer" generation of experimental vaccines have often been composed of synthetic, recombinant, or highly purified peptide subunit antigens and rely on this fact to stimulate highly specific immune responses (Fitchen *et al.*, 1995; Durrani *et al.*, 1998., Oscherwitz *et al.*, 1999; Staczec *et al.*, 2000; Liao *et al.*, 2000 and 2004). In comparison, whole-killed and live-attenuated vaccines, which are not deemed safe as they pose the threat of reverting to a wild-type form, elicit broader and possibly more non-specific immune responses. Purified subunit vaccines, however, are known to stimulate weaker, localized immune responses as they lack the immunomodulatory (enhancing) components associated with whole-killed and live-attenuated vaccines (www.niaid.nih.gov/daids/vacine/adjuvants/htm). Immunostimulatory (enhancing) compounds or adjuvants are therefore often administered in conjunction with these subunit vaccines. Adjuvants are defined as compounds that can enhance the immune response to a particular antigen when incorporated into the inoculum. Adjuvants encourage prolonged immune responses by allowing the slow or intermittent release of antigen from the site of inoculation, which otherwise would be rapidly cleared from the inoculation site. Adjuvants also assist with delivering the antigen to antigen-presenting cells (APCs) in the spleen and lymph nodes where most necessary antigen processing occurs, and most necessary cell-cell interactions occur for the generation of plasma cells. A third mechanism by which adjuvants enhance immune responses is by activating various cells involved in the immune response by means of surfactants. Surfactants commonly constitute one or more components of all adjuvants and usually consist of bacteria, bacterial products or derivatives of bacterial products (www.nal.usda.gov.awic/pubs/antibody/overview.htm). Thus depending on the formulation, adjuvants can activate different pathways of the immune response.

There are currently several different types of adjuvants in use for animal immunogenicity analysis which are able to stimulate different parts of the immune system (www.nal.usda.gov.awic/pubs/antibody/overview.htm). Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) appear to be the most frequently used adjuvants. FCA is primarily composed of a water and mineral oil emulsion containing killed mycobacteria. FCA is known to cause severe chronic inflammatory responses at the site of inoculation, which may cause pain and discomfort to the inoculated animal (www.nal.usda.gov.awic/pubs/antibody/overview.htm). FIA is identical in composition to FCA except lacks killed mycobacteria, and consequently causes inflammatory reactions which are much less severe than FCA (www.nal.usda.gov.awic/pubs/antibody/overview.htm). FIA is also known to favour humoral immune responses without any cell mediated immune responses, which made it ideal for my purposes in this study (www.nal.usda.gov.awic/pubs/antibody/overview.htm).

Vaccine delivery can potentially occur via a number of different routes, and each route has previously been used to elicit a desired immune response. Oral, nasal or rectal delivery of a vaccine has been shown to stimulate mucosal immune responses (Awram *et al.*, 2002; Koo *et al.*, 1999; Marusic *et al.*, 2001; Durrani *et al.*, 1998), while direct inoculation of animals subcutaneously, intramuscularly or intradermally is known to stimulate more generalized systemic immune responses (Liao *et al.*, 2004 and 2000; Letvin *et al.*, 2001; Staczek *et al.*, 2000; Koo *et al.*, 1999; Joelsen *et al.*, 1997). For HIV it is most desirable to stimulate a disseminated mucosal immune response, as mucosal surfaces in the genital regions are the most common routes of viral acquisition. In addition, protective systemic

(cellular/humoral) immune responses would also be desired to complete the barrier of protection against HIV infection.

In this chapter the core objectives of this project were investigated. Are the recombinant TMV particles immunogenic and capable of stimulating a V3 peptide-specific humoral immune response? Of greater significance, is whether a V3 peptide-specific response is observed; will the serum of inoculated animals display an ability to neutralize an HIV infection? Guinea pigs were selected as the animal of choice in these experiments as their sera contain lower levels of background HIV-1 NAb activity (personal communication, Dr David Montefiori; Liao *et al.*, 2000; Zhu *et al.*, 2003; Liao *et al.*, 2004; Gao *et al.*, 2005). Thus, selected TMV recombinants were inoculated subcutaneously in conjunction with Freund's incomplete adjuvant into guinea pigs and serum extracted from these animals was tested for the desired responses.

As a result of time constraints not all recombinant TMV constructs were assessed for their ability to stimulate an immune response in the selected guinea pig model. Thus, only the pLSB2109+151a (100µg/site TMV), pLSB2109+151b (100µg/site TMV) and a combination of pLSB2109+151a (50µg/site TMV) and pLSB2109+151b (50µg/site TMV) recombinant TMV constructs were assessed for their ability to stimulate an anti-V3 peptide response and compared to the pLSB2109 non-recombinants. Although it does seem contradictory to use this particular construct in light of what has been discussed in chapter 3, the selection of this recombinant vector was determined before the actual quantitive data in chapter 3 was generated.

4.2 MATERIALS AND METHODS

The Dunkin Healy strain of guinea pigs was used in all immunization experiments. All guinea pigs used were female and averaged between 500g and 600g in weight.

4.2.1 Guinea pig inoculation procedure and schedule

Four sets of guinea pigs were immunized with different TMV constructs and each set consisted of four guinea pigs. Each animal was injected subcutaneously at 4 sites along the neck and lumbar regions with 100ug/site of a 50% volume/volume mixture of Freund's incomplete adjuvant (Sigma-Aldrich) and TMV preparation in a phosphate buffer (PBS). The guinea pigs were inoculated at 4 week intervals and bled under anaesthesia (65% (100mg/ml) Ketamine + 35% (2%) Rompun mixture) via cardiac bleed yielding 4 – 5 ml blood/500g guinea pig prior to each inoculation. Effectiveness of the anaesthetic was evaluated by checking the pedal and eye reflexes of the animal before attempting the cardiac bleed. Serum was separated from the cellular constituents in the blood by centrifuging the blood samples at 3000g for 15 minutes. Serum samples were stored at 4 ^{o}C.

4.2.2 V3 peptide-specific serum ELISA

ELISAs were conducted according to the protocol outlined in Appendix A(IX). Initially, ELISA plates were coated with 100ng of a V3 peptide per well. I used the same V3 peptide as the ELISA described in 3.2.5 i.e. N-(CTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHCN)-C. Plates were probed overnight with a 1/100 dilution of serum from inoculated guinea pigs. Presence of V3 peptide-specific antibodies was then confirmed by probing the ELISA plates

with a 1/10000 dilution of alkaline phosphatase conjugated goat anti-guinea pig secondary antibody (Sigma-Aldrich).

4.2.3 HIV Neutralization assays

As a result of the complexity, lack of facilities, required equipment and expertise required to conduct the neutralization assay, at my current institution (UCT) I could not conduct the assay personally. Neutralization assays were therefore conducted by Natasha Taylor at the National Institute for Communicable Diseases, Johannesburg, South Africa. Serum samples from immunized animals were tested for neutralizing antibody activity using a single cycle recombinant pseudovirus assay (Montefiori., 2004). This assay measures neutralization as a function of a reduction in luciferase reporter-gene expression in HeLa-derived TZM-bl indicator cell line. This cell line expresses high levels of CD4 and CCR5 along with endogenously expressed CXCR4. Luciferase activity is quantified by luminescence and is directly proportional to the number of infectious pseudoviruses (Du151) infecting TZM-bl cells.

Serum samples from 4 guinea pigs (animals' no. 712, 725, 772, 791) at three time points were selected (pre-bleed and 1st and 2nd bleed serum samples). Samples were heat-inactivated at 56^{o} C for 15 minutes. Assays were conducted in flat-bottomed 96 well microtitre plates. One hundred microlitres of Dulbecco's modified Eagles medium +10% fetal calf serum (Growth medium/GM) was added to each well. Each serum sample (22µl) was then added in triplicate to the wells of the microtitre plate as 1/10, 1/30, 1/90, 1/270 dilutions. Fifty microlitres of the test virus (Du151 derived pseudovirus) was then added to each well. Plates were then

incubated at 37°C in a humidified incubator for 1 hour. One hundred microlitres of the TZM-bl target cells to be infected were then added ($1x10^4$/well) to each well. Plates were then incubated at 37°C in 5% CO_2 for 48 hours. One hundred and fifty microlitres of culture medium was then discarded from each well. Following this 100µl of Bright Glo™ reagent was dispensed into each well and plates were incubated at room temperature for 2 minutes to allow complete cell lysis. Contents of each well were mixed by pipette action (two strokes) and 150µl transferred to a corresponding 96-well black plate. Luminescence was then read from the plate immediately in a luminometer. The 50% infectious dose (ID50) was then calculated (the reciprocal dilution where 50% of the virus is neutralized by the serum sample). Wells with RLU <2.5 times background were considered negative for the calculation.

A positive control was run on each plate. The positive control used was IBU-21, which is a plasma sample from an HIV-1 infected individual with a high titre of neutralizing antibodies. This reaction consisted of 45ul GM plus 5ul IBU-21 in triplicate. For this control a series of 4, 3-fold dilutions, 1/45, 1/135, 1/405, 1/1215 was made. The no virus, cell control consisted of only 150µl GM with 100µl of TZM-bl cells ($1x10^4$/well). The serum free, virus control was made up identically to the experimental samples, except no serum was added.

4.3 RESULTS

4.3.1 V3-specific Serum Response assay

V3 peptide-specific serum ELISAs all indicated that a V3 peptide-directed immune response was stimulated by the inoculated recombinant TMV constructs (Figures 4.1, 4.2, and 4.3). Serum extracted from the pLSB2109 non-recombinant inoculated guinea pigs all displayed very similar results and thus as a point of reference absorbance readings were averaged and displayed on each of the graphs below to serve as the negative control. As some serum samples reacted very strongly in the ELISA a maximum absorbance cut-off of 2 ODU was set to allow comparisons between the various serum samples. It was evident that pre-bleed serum samples extracted before the first inoculation, as well as all serum extracted from guinea pigs inoculated with the non-recombinant TMV constructs, all displayed only background level reactions and thus did not display any V3 peptide-specific immune responses (Figures 4.1, 4.2, and 4.3). The V3 peptide-specific immune responses were found to noticeably increase after each boost and demonstrated the strongest response at the second bleed stage (Figures 4.1, 4.2, and 4.3).

Serum extracted from guinea pigs inoculated with the pLSB2109+151a recombinant construct did not display uniform V3 peptide-specific immune responses (Figure 4.1). Results were found to vary considerably between guinea pigs, and serum from guinea pigs 771 and 773 only displayed relatively significant reactions for the second bleed, while prior samples displayed reactions comparable to the negative control. These guinea pigs though may have been

immunocompromised as they had diarrhoea, which may have caused the weaker V3 peptide-specific serum response. V3 peptide-specific responses in the serum extracted from guinea pigs 770 and 772 on the other hand were clearly stronger than those displayed in 771 and 773.

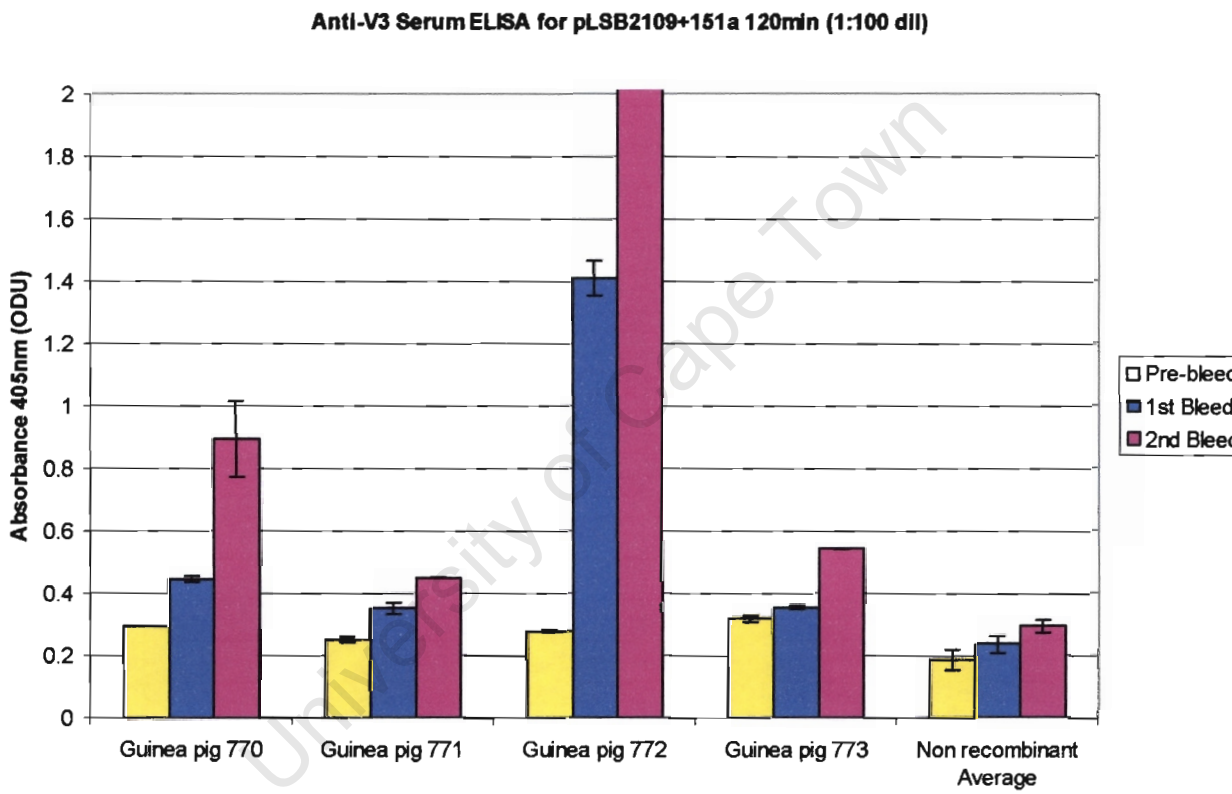


Figure 4.1 V3 peptide-specific ELISA result for serum isolated from guinea pigs 770, 771, 772 and 773 at pre-bleed, 1st-bleed and 2nd-bleed stages relative to inoculation with the pLSB2109+151a TMV recombinant. For comparative purposes the average result for sera isolated from guinea pigs inoculated with the pLSB2109 non-recombinants is displayed.

Sera extracted from the pLSB2109+151b recombinant construct-inoculated guinea pigs displayed more uniform set V3 peptide-specific immune responses with no particular guinea pig's serum extraction displaying an exceedingly positive or negative result (Figure 4.2). ELISA results for this set of inoculated guinea pigs

clearly indicates that the desired V3 peptide-specific immune response has been stimulated.

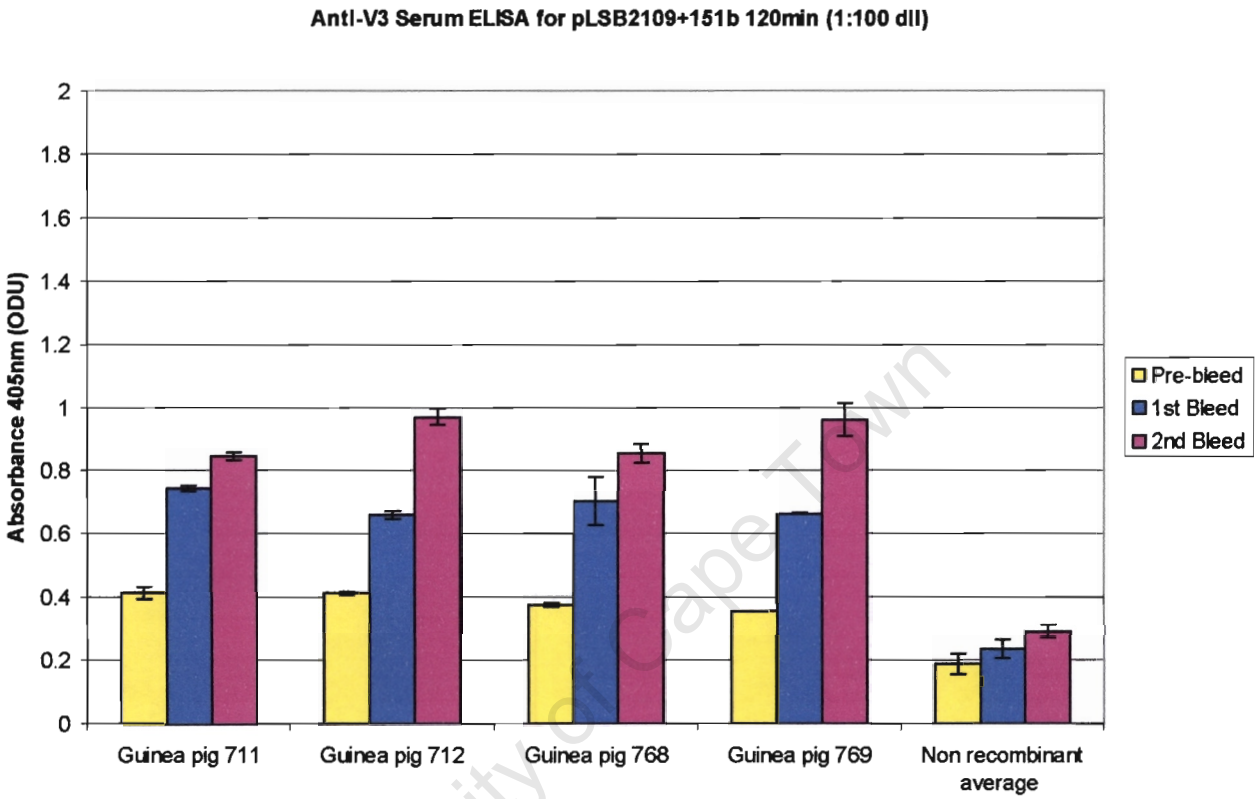


Figure 4.2 V3 peptide-specific ELISA result for serum isolated from guinea pigs 711, 712, 768 and 769 at pre-bleed, 1st-bleed and 2nd-bleed stages relative to inoculation with the pLSB2109+151b TMV recombinant. For comparative purposes the average result for sera isolated from guinea pigs inoculated with the pLSB2109 non-recombinant is displayed.

Similar to the serum extracted from the pLSB2109+151b inoculated guinea pigs, the sera extracted from guinea pigs inoculated with both pLSB2109+151a and pLSB2109+151b recombinants give uniform results with no particular serum extraction displaying an exceedingly positive or negative result (Figure 4.3). A V3 peptide-specific immune response was nevertheless observed for all of this group's inoculated guinea pigs, which varied between animals. Serum extracted

from guinea pig 725 in particular displayed the strongest V3 peptide-specific immune response.

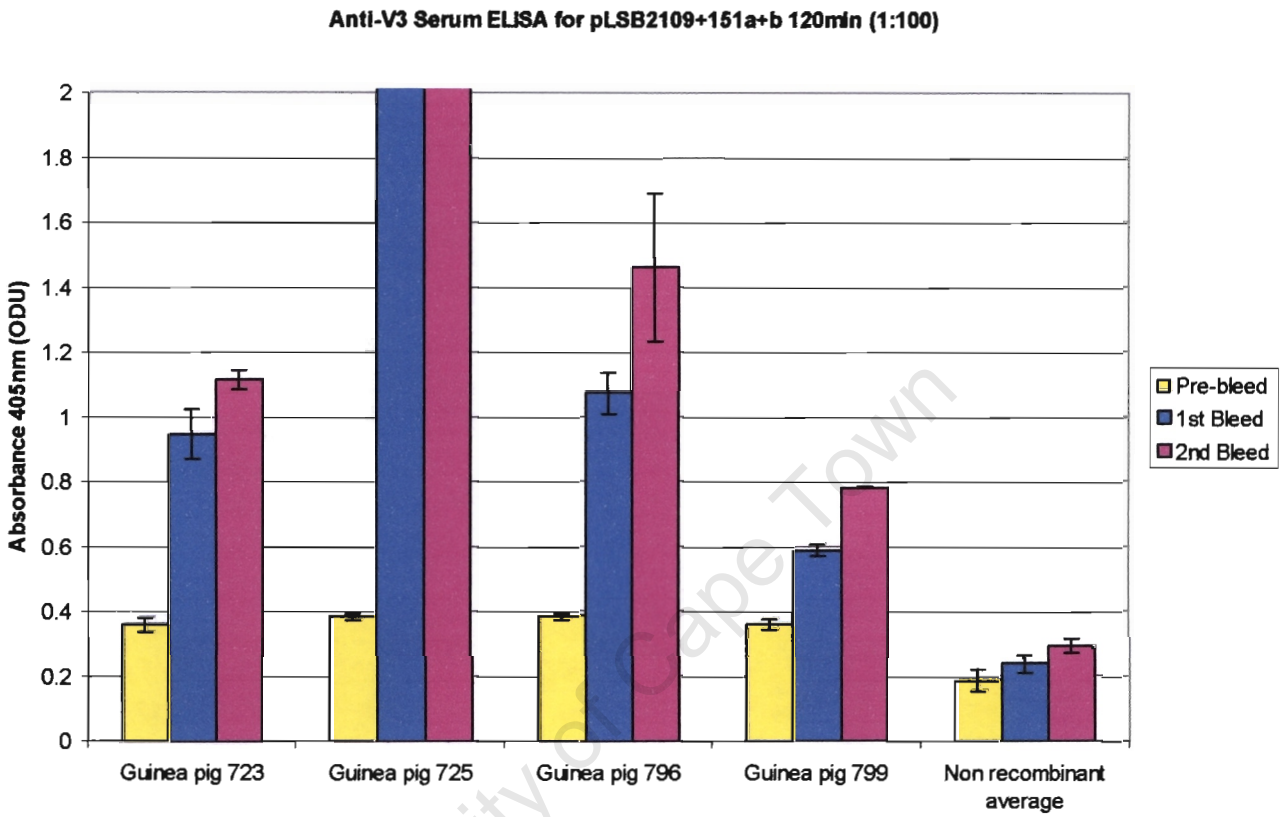


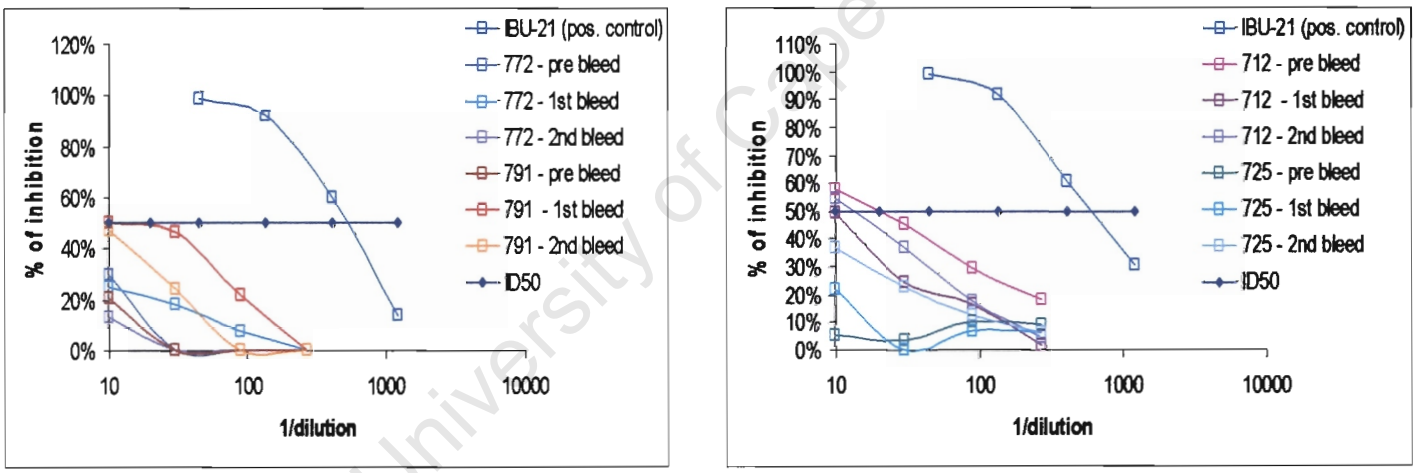
Figure 4.3 V3 peptide-specific ELISA result for serum isolated from guinea pigs 723, 725, 796 and 799 at pre-bleed, 1st-bleed and 2nd-bleed stages relative to inoculation with the pLSB2109+151a and pLSB2109+151b TMV recombinants. For comparative purposes the average result for sera isolated from guinea pigs inoculated with the pLSB2109 non-recombinants is displayed.

4.3.2 Serum Neutralization Assays

To assess whether the serum that displayed the V3 peptide-specific immune responses was capable of neutralizing an HIV infection, serum samples that displayed the strongest responses in the ELISAs were used in HIV neutralization assays. Serum extracted from guinea pigs 772, 712 and 725 were tested for the ability of the pLSB2109+151a, pLSB2109+151b and pLSB2109+151a and 151b combination respectively, to stimulate a NAb response. Also, serum extracted from

a guinea pig (791) inoculated with the pLSB2109 non-recombinant was tested to serve as a negative control.

The selected serum samples were all subjected to an HIV neutralization assay in replicate, but despite the observed V3 peptide-specific activity none of the serum samples displayed an ability to neutralize HIV-1 infection (Figures 4.4(a) and (b)). All tested serum samples thus displayed results indistinguishable from observed background activity observed for the negative control (791) (Figure 4.4(a)) and incomparable to results generated by the positive controls.



(a) (b)

Figure 4.4. Neutralization assay results, (a) results for serum extracted from guinea pigs 772 and 791 inoculated with pLSB2109+151a and the pLSB2109 non-recombinant respectively at the pre-bleed, 1st bleed and 2nd bleed stages, (b) results for serum extracted from guinea pigs 712 and 725 inoculated with pLSB2109+151b and the pLSB2109+151a and 151b at the pre-bleed, 1st bleed and 2nd bleed stages.

The positive control, IBU-21 (serum sample with high titre of neutralizing antibodies) as expected showed inhibition of viral infection that decreased as the antibody concentration was diluted (Figures 4.4(a) and (b)). The ID50 titers

(indicated by solid black line) were 1: 515 and 1: 599 for the 2 positive controls run on each plate which is within the acceptable range. None of the guinea pig serum samples therefore showed any neutralization activity. The low level neutralization activity seen in the pre-bleed of guinea pig 712 is most likely toxicity of serum at the highest concentration (figure 4.4(b)).

4.4 CONCLUSION

The concluding component of this study encountered both its share of success and failure in relation to the immunological analyses. The first important point is highlighted by the fact that the recombinant TMV-derived vector was capable of stimulating and efficiently targeting a desired immune response to the foreign protein. This was evinced by the strong V3-specific immune response stimulated in the inoculated guinea pigs. On average, immune responses stimulated by the different recombinant vectors did not vary much and the overall strong V3-specific immune responses could be attributed to the effective means of epitope presentation by each of the TMV-based vectors. Variations in the immune responses did occur though, notably with the pLSB2295 recombinant vector. Moreover, weakened V3-specific immune responses could be attributed primarily to the health of the guinea pig, some of which were possibly immunocompromised (771, 773).

Despite the strong V3-specific immune responses observed in the serum of inoculated guinea pigs, these can be regarded as ineffectual, as the stimulated immune responses did not confer any HIV neutralization activity. As observed in figures 4.4(a) and (b) none of the serum samples generated results that could be distinguished from the negative control (serum extracted from guinea pig inoculated with the pLSB2109 non-recombinant). A number of reasons could be presented for the failure to neutralize the Du151 derived pseudovirus infection of target cells in the neutralization assay. Popular consensus suggests that the Du151 primary isolate from which the pseudovirus was derived, aided in the failed virus neutralization. Several studies have demonstrated that V3-specific antibodies

on the envelope glycoproteins of primary isolates can be inaccessible for antibody binding (Beddows *et al.*, 1998; Spenlehauer *et al.*, 1998, Letvin *et al.*, 2001; Parren *et al.*, 1998; Zhu *et al.*, 2003; Gorny *et al.*, 1998). Hence V3-specific antibodies have been inconsistent in their neutralization of primary isolates. This may seem contradictory as the Du151 primary isolate-derived V3 epitope used was selected as a result of the broad cross-neutralizing activity displayed in the infected individual. Letvin *et al.*, (2001) has demonstrated similarly in SHIV models that V3 in some primary isolates may only be a partially effective target for neutralizing antibodies induced by peptide immunogens compared to other primary isolates. The context in which the V3 epitope was presented to the immune system may also have contributed to the neutralization failure. The linear V3 epitope displayed by the recombinant TMV could have stimulated an inferior neutralization response as compared to the native three dimensional structure of the V3 loop. The display of T-helper epitopes has been shown to affect the overall immunogenicity of an epitope (Grudner *et al.*, 2004). The three dimensional positioning of the T-helper epitopes within the native gp120 may therefore have affected antigen processing. As described earlier, the binding of anti-V3 antibodies is most effective when the V3 domain retains its conformation and with respect to this the strength of binding of anti-V3 antibodies to HIV virions has been shown to correlate with neutralizing activity, which has also been described for other epitopes (Schreiber *et al.,* 1997; Krachmarov *et al.,* 2001; Gorny *et al.,* 2002; Burrati *et al.*, 1998; Taylor *et al.*, 2000).

Traditionally the strength of an immune response has not always been reflective of the neutralization efficiency of the immune response to an HIV infection. Such

immune responses have been displayed in studies investigating the ability of C4-V3 chimaeras to neutralize an SHIV infection (Letvin *et al.*, 2001; Liao *et al.*, 2000). C4-V3 chimaeras displaying V3 peptide derived from SHIV89.6 or 89.6P were used to immunize animals and serum samples were assayed for V3-specific as well as neutralizing antibody activity. Both SHIV89.6 and 89.6P derived C4-V3 constructs elicited strong V3-specific serum antibody responses. Antibody responses generated by the SHIV89.6 C4-V3 fusions were capable of stimulating antibody levels capable of recognising both 89.6-V3 and 89.6P-V3. While the V3-specific antibodies generated by the SHIV89.6 C4-V3 fusions neutralized SHIV-89.6 but not SHIV-89.6P despite the fact that V3-specific antibodies recognised both viruses equally well. Immunization with the SHIV89.6P on the other hand generated antibodies capable of neutralizing both SHIV89.6 and SHIV89.6P. Although the magnitude of SHIV89.6 neutralization exceeded the neutralization of 89.6P. These observations suggest that the V3 loop on some primary patient HIV-1 isolates may only be a partially effective target for neutralizing antibodies induced by peptide immunogens and would seek to discredit the notion that the antibody response generated by the TMV chimaeras failed to neutralize HIV pseudovirion activity as a result of an overall weak immune response.

Possibly an alternative means of epitope display would be better suited to the display of V3. One such display system utilises the Alfalfa mosaic virus CP on a TMV based backbone, which has allowed for the insertion of much larger epitopes than can be accommodated by the TMV CP (Yusibov *et al.*, 1997). This vector system which still utilises the TMV mechanism of gene expression would therefore retain the native structure of V3 and presumably stimulate a better neutralizing

response in infected individuals. It may also be possible therefore that an unidentified region of V3 on primary isolates, which remains exposed for the initial contact with CCR5/CXCR4 receptors serves as the target for neutralizing antibodies.

CHAPTER 5

Final Conclusion

Now, more than ever, there is a pressing need for the development of a protective/preventative measure against HIV infection. Infection rates, particularly in sub-Saharan Africa, have reached colossal proportions and are still accelerating. Internationally, billions of dollars are invested/required each year for antiretroviral treatment of infected individuals with a projected need of US $2.8 billion dollars required for 2005 to negate the further spread of infection (http://www.who.int/3by5/progressreport05/en/). The recent tsunami that struck South-East Asia in December 2004 has killed in excess of 200000 people and has resulted in one of the largest international humanitarian efforts. Countries have collectively pledged in excess of US $4 billion for this disaster, and despite this, the mobilisation of funds for purposes of HIV treatment or research which on average has the potential to save a far greater number of people, has not received such wide appeal for funding.

Currently in developing countries the lowest price for the treatment of a single infected individual is around US $140 per person per year, but average prices are at least US $300 (http://www.who.int/3by5/progressreport05/en/). This is not good news as the poorest countries concentrated in sub-Saharan Africa and South-East Asia appear to be affected the worst by the AIDS pandemic. As these countries are not likely to cope financially with the treatment of infected individuals without foreign aid, the pricing of the drugs appears to pose a barrier to availability of

antiretroviral treatment. It has also become apparent that investment in HIV infection prevention (vaccines) should ultimately be more sensible and cost effective than the partially effective antiretroviral treatment of infected individuals currently used. This study therefore does not dispute the importance of continued antiretroviral therapy, but does support the use of an alternative, protective strategy against HIV infection.

The requirement for an alternative, cost-effective means of treatment (vaccines) has advocated the use of plants as bioreactors. Plants used in concert with recombinant plant viral vectors, have the potential to greatly reduce the costs of raw materials, eliminate the requirement for highly specialized facilities and can be rapidly scaled-up to produce large quantities of the desired product. Today the use of plants also appears to have greater social appeal and encounters greater social acceptance as it would not harbour any human pathogens.

This project has successfully yielded a polymeric epitope display system utilising a set of TMV-based expression vectors. Vectors successfully displayed the HIV-1 subtype C V3 epitope at either the N-terminus, C-terminus or 60S-loop positions of the TMV CP. The assembled virions in turn, displayed the foreign epitopes at their surface, which changed the physiochemical properties and predicted size of the virus. Despite the physiochemical alterations to the assembled virion (increased virion length and coat protein charge), plants infected with the recombinants showed leaf shrivelling and growth stunting symptoms similar to that of wt TMV and similarly did not cause excessive necrosis in the infected plant hence yielded good yields during extraction. Recombinant TMV CP yields depended on the

position at which the foreign epitope was inserted in the coat protein. Insertion of the V3 epitopes into the 60S-loop position generated the most stable and abundant recombinant CP. N-terminal insertions interfered with expression levels and the C-terminal insertions generated unstable CP recombinants that reverted to a wt TMV form.

Immunogenicity of the C-terminal CP recombinants was assessed in a guinea pig animal model. All recombinant vectors inoculated into guinea pigs stimulated a strong V3-specific serum antibody response successfully. This indicated that the recombinant vectors were effective at displaying the foreign epitope to the immune system and enabled a targeted immune response towards the V3 epitope. Serum extracted from the inoculated guinea pigs was tested for any HIV neutralization activity utilising a primary isolate-derived pseudovirus, but unfortunately none of the serum samples even at a low serum dilution exhibited any neutralization activity. As discussed previously a number of reasons could have accounted for this. This barrier to HIV primary isolate neutralization has been encountered in several other studies, which prompts further studies with respect to the barriers of primary isolate neutralization (Letvin *et al.*, 2001; Zhu *et al.*, 2003; Messer *et al.*, 2004). The complete absence of neutralization activity clearly challenges reasoning for using the Du151-derived V3 epitope which as discussed was chosen as a result of the broad cross-neutralizing activity displayed in the infected individual. A study in which V3-specific immune responses were only found to be partially effective against some primary isolates has suggested that protection against viral challenge may be conferred by an antibody activity other than neutralization (Letvin *et al.*, 2001). This group has also suggested that protection *in*

vivo may arise from a barely detectable NAb, which is diluted during *in vitro* analyses. Non-NAbs analysed using the Friend retrovirus have been shown to aid an immune response and thus should not be discredited (Messer *et al.*, 2004). Passive transfer of non-NAbs along with NAbs to B-cell deficient mice was found to stimulate a far superior response compared to the transfer of the NAbs alone. The enhanced immunogenicity is likely a result of an additional cellular immune responses promoted by the non-NAbs. It is also therefore realised that the stimulation of both a humoral as well as a cellular immune response is necessary to effectively neutralize an HIV infection. In the Friend retrovirus model vaccine primed T-cells were found to work synergistically with NAbs to provide a level of protection greater than that provided by either NAbs or T-cells alone (Messer *et al.*, 2004).

To conclude, it is clearly evident that the TMV-based vector system used was highly successful in its ability to stimulate a strong immune response towards the foreign epitope it presented. The problem encountered was that the stimulated response did not include the desired ability to neutralize an HIV infection and for this the particular selected V3 epitope could be held accountable. This suggests that if an appropriate HIV envelope protein epitope were selected, the TMV-based vector system could still prove useful and this study would have paved a direct path towards the development of variants of my recombinants. The applications of this vector also extends beyond HIV vaccine development and could be used for the presentation of epitopes derived from other disease-causing organisms such as *Plasmodium* species, Influenza virus, Hepatitis virus and *Mycobacterium tuberculosis*.

HIV research, and in particular the development of a protective vaccine has become one of the biggest, most well-funded and ambitious endeavours that the international scientific community has ever embarked on. Unlike many other vaccine types the HIV vaccine has proven highly elusive as the virus has surpassed all preconceived notions as to its abilities and with that every strategy we have employed to eradicate it. Hence the continued research into all aspects of HIV is paramount to the discovery of an effective neutralization strategy.

APPENDIX A

Standard Methods

I. Bacterial growth media, conditions, antibiotics and strains

All bacterial cultures were grown in Luria-Bertani (LB) broth (5g bacto-tryptone, 5g yeast extract, 10g NaCl, 950ml distilled H_2O, pH adjusted to 7.4 using NaOH, made up to 1L and autoclaved.

Luria-Bertani agar was made up with the same constituents as LB, but also contained 15g agar/L.

Ampicillin was used as a selective marker at 100μg/ml concentrations in both LB media and Luria agar to select for the ampicillin-resistant clones.

Liquid cultures were all grown at 37°C with agitation.

The DH5α strain of *E.coli* (Invitrogen) was used for the transformation of DNA.

II. DNA extraction procedures

a. Small-scale plasmid DNA extraction

DNA was isolated from 5ml overnight bacterial cultures according to the protocol of the QIAprep Spin Miniprep Kit (Qiagen), which is based on the alkaline hydrolysis technique of Sambrook *et al.* (1998).

b. Large-scale plasmid DNA isolation

DNA was isolated from 100ml overnight bacterial cultures using and carried out according to the protocol of the Qiagen Plasmid Midi Kit (Qiagen), which is based on the alkaline hydrolysis technique of Sambrook *et al.* (1998).

III. Shrimp alkaline phosphatase DNA treatment

Restriction enzyme-digested DNA was treated with 1u/μl of Shrimp Alkaline Phosphate (Roche) for 30 minutes at 37°C, and the reaction was terminated by incubation at 65°C for 10 minutes to remove 5'-phosphate groups to prevent vector self-ligation.

IV. Agarose gel electrophoresis of DNA and RNA

Gel electrophoresis of both DNA and RNA was carried out using 1% agarose gels containing 0.5μg/ml of ethidium bromide in a 1x Tris-borate EDTA buffer at 95 volts. DNA fragment size was estimated using a λ-DNA marker digested with *PstI* giving a size estimation range of between 11497bp and 15bp. For RNA gels, all reagents were prepared under RNase free conditions.

V. Gel and endonuclease reaction purification of DNA

Purification of DNA from agarose gels or directly from endonuclease reactions to remove undesired buffers and enzymes was carried out using the Amersham Biosciences DNA purification kit. To isolate specific DNA fragments derived from endonuclease reactions, the DNA was loaded onto a 1% agarose gel containing 0.5μg/ml ethidium bromide and electrophoresed at 95 volts. The bands of interest were visualized on a UV transilluminator plate (310nm) and excised using a sterile scalpel blade. The excised gel slice was placed into a 1.5ml eppendorf tube and the DNA extraction was carried out according to the method outlined in the above-mentioned kit.

VI. Transformation

DH5α *E. coli* cells were made competent and transformation was carried out using the method of Chung and Miller. (1989). Transformations using 100μl of competent cells with 2μl of pGEM®-T Easy (Promega) ligation reaction DNA or 50ηg of DNA was conducted. The DNA and competent cells were mixed in a 1.5 ml eppendorf tube and chilled on ice for 30 minutes. The cells were then heat-shocked at 37°C for 25 seconds, followed by incubation on ice for 2 minutes. 700μl of LB was then added to the transformation mix, which was then incubated at 37°C for 1 hour 30 minutes. 100μl volumes of each transformation mix was then plated on Luria agar plates containing 100 μg/ml ampicillin as a selective marker. Transformants were plated on Luria agar plates that also contained 800μl (10%) Xgal and 2ml (0.1M) IPTG/400ml Luria agar.

VII. DNA sequencing

Purified miniprep DNA was used for the sequencing reactions by means of a technique derived form the dideoxy chain terminating method of Sanger *et al.* (1977). This service was provided by the Department of Molecular and Cell Biology's DNA sequencing service, University of Cape Town, Cape Town, South Africa, which used the Thermosequence™ cycle sequencing kit (Amersham Biosciences) and reaction products analyzed on an ALF-Express™ DNA Sequencer version AMV2.0 (Pharmacia Biotech). Analysis of the generated sequences was done using the DNAMAN program for Windows version 4.13 (Lynnon BioSoft, © 1994-1999).

Sequencing primers used:

For pGEM-T® Easy – pUC/M13 Primer, Forward (24mer) (Promega)

For pLSB vectors -TMV coat protein forward: 5' AGG CTA CTG TCG CCG AAT 3'

- TMV coat protein reverse: 5' GCG TTA TCG TAC GCA CCA 3'

VIII. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described by Laemmli. (1970). Approximately 1.5mm thick gels comprised of a 15% resolving gel and a 4% stacking gel were poured into a Hoeffer SE-600 vertical slab gel apparatus (Hoeffer Scientific Instruments). Samples to be electrophoresed were denatured by mixing with an equal volume of protein loading buffer (10% [w/v] SDS, 15% glycerol, 15% 2-mercaptoethanol, 0.01% bromophenol blue, 0.125M Tris-HCl pH6.8) and heating to 100°C for 10 minutes. Electrophoresis was carried out at 20mA/gel until the dye front reached the bottom of the gel. Gels were stained with 0.2% (w/v) PAGE blue 83 (BDH chemicals) dissolved in a 45:45:10 (v/v) mixture of methanol, water and glacial acetic acid. Stained gels were destained with a destain solution consisting of 65:25:10 (v/v) mixture of methanol, water and acetic acid.

IX. Western Blot

Protein from SDS-PAGE gels was transferred onto nitrocellulose membranes (Hybond) using a semi-dry blotting electroblotter (BioRad™). Blotting was carried out for 40 minutes at 15 volts, after which membranes were blocked for 30 minutes at room temperature in a 1% Tween-20, 5% milk powder in phosphate buffered saline solution. Depending on the type of blot, membranes were next probed overnight in an appropriate dilution of primary antibody at 4°C. Blots were

subsequently washed with blocking buffer 4 X for 15 minutes/wash before being probed with an appropriate dilution of alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich) for 2 hours at room temperature. The blots were next washed with blocking buffer lacking milk powder as above. Detection was via a colorimetric reaction using the **SIGMA*FAST*™,** 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT) containing tablets as per supplier's directions (Sigma-Aldrich).

X. Direct ELISA

The direct ELISA protocol is based on the technique of Ausubel *et al.* 1994. One hundred microlitre volumes of relevant protein samples were used to coat 96 well Maxisorp® ELISA plates (Nunc), which were then incubated overnight at 4°C. Plates were washed 3x using 300µl PBS per well with an ELISA plate washer (Titretek® Microplate washer S8/12). Two hundred microlitres of blocking buffer (Appendix A(IX)) was added to each well and incubated for 2 hours at room temperature. Plates were then washed 3x using 300µl PBS per well. Plates were probed overnight at 4°C with appropriate dilutions of protein-specific primary antibodies diluted in blocking buffer. Plates were then washed 3x using 300µl PBS per well. One hundred microlitre volumes of a 1/10000 dilution of an alkaline phosphatase-conjugated secondary antibody diluted in PBS containing 3% BSA was added to each well and incubated at 37°C for 2 hours. Plates were washed 3x using 300µl PBS per well. One hundred microlitres of p-nitrophenyl phosphate (PNPP) substrate (1mg/ml), made fresh using SIGMA FAST™ p-nitrophenyl tablet sets was added to each well (Sigma Aldrich). Absorbance readings were read at

405nm using the Titretek Multiskan® Plus MKII ELISA plate reader 2 hours after adding the substrate.

Appendix B

Vectors used

1. pGEM®-T Easy DNA vector (Promega)
2. pLSB2109 Vector map (LSBC)
3. pLSB2109 coat protein sequence
4. pLSB2295 Vector map (LSBC)
5. pLSB2295 coat protein sequence
6. pLSB2296 Vector map (LSBC)
7. pLSB2296 coat protein sequence

(1)

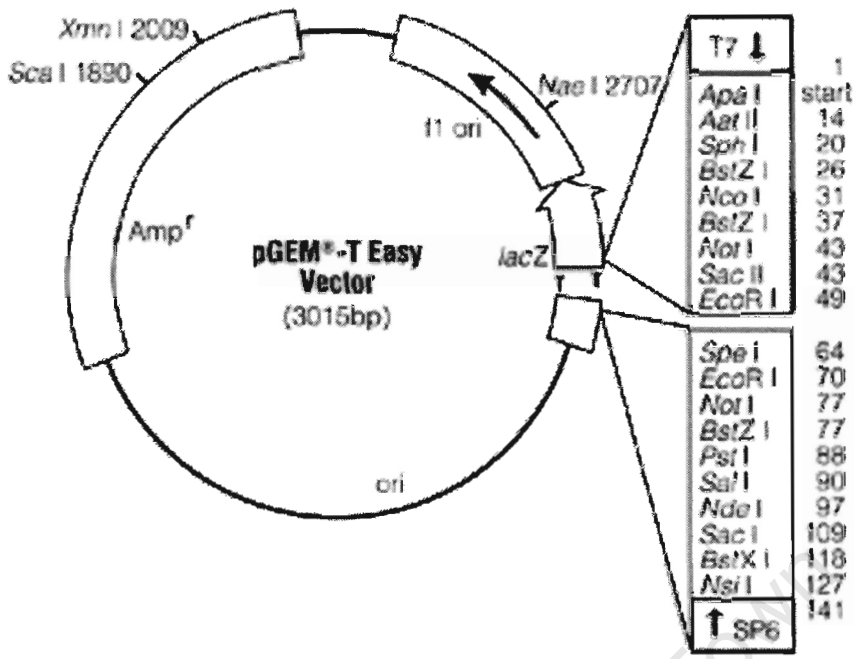


Figure B1 The pGEM®-T-easy cloning vector (Promega) used for the cloning of the V3-loop epitopes into the TMV based vectors.

(2)

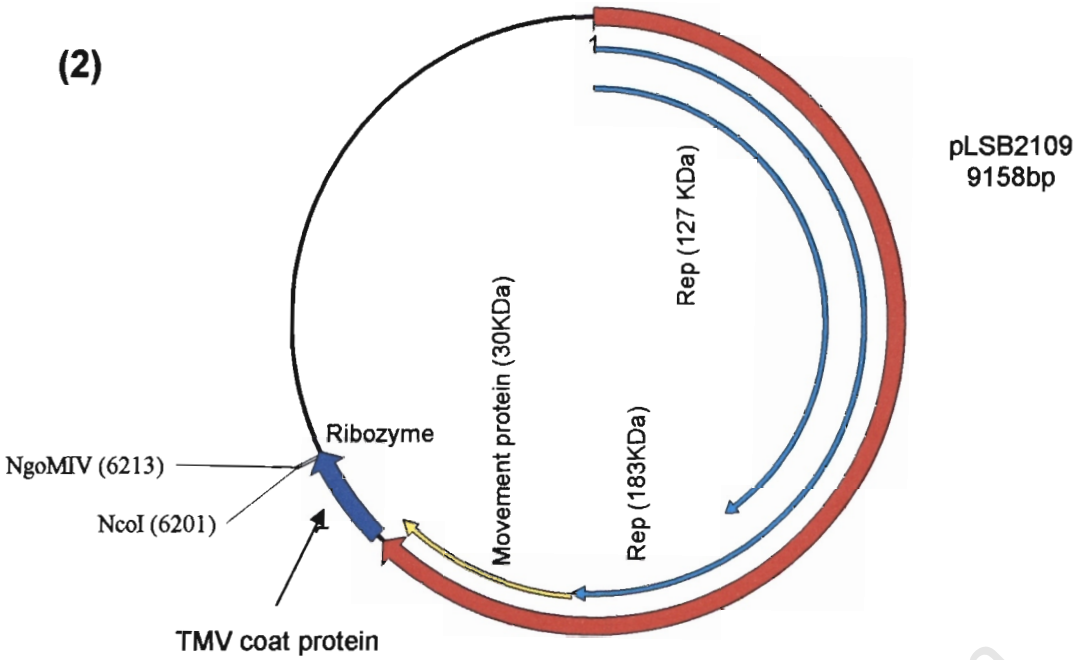


Figure B2 pLSB2109 TMV based vector provided by LSBC, which contains the NgoMIV/ NcoI cloning site at the C-terminus of the TMV coat protein.

(3) pLSB2109 coat protein sequence

Translation of pLSB2109(1-519)
Total amino acid number: 172, MW=19075
Max ORF starts at AA pos 1(may be DNA pos 1) for 172 AA(516 bases), MW=19075

```
1          ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCATCAGCGTGGGCCGAC
1           M  S  Y  S  I  T  T  P  S  Q  F  V  F  L  S  S  A  W  A  D

61         CCAATAGAGTTAATTAATTTATGTACTAATGCCTTAGGAAATCAGTTTCAAACACAACAA
21          P  I  E  L  I  N  L  C  T  N  A  L  G  N  Q  F  Q  T  Q  Q

121        GCTCGAACTGTCGTTCAAAGACAATTCAGTGAGGTGTGGAAACCTTCACCACAAGTAACT
41          A  R  T  V  V  Q  R  Q  F  S  E  V  W  K  P  S  P  Q  V  T

181        GTTAGGTTCCCTGACAGTGACTTTAAGGTGTACAGGTACAATGCGGTATTAGACCCGCTA
61          V  R  F  P  D  S  D  F  K  V  Y  R  Y  N  A  V  L  D  P  L

241        GTCACAGCACTGTTAGGTGCATTCGACACTAGAAATAGAATAATAGAAGTTGAAAATCAG
81          V  T  A  L  L  G  A  F  D  T  R  N  R  I  I  E  V  E  N  Q

301        GCGAACCCCACGACTGCCGAGACGTTAGATGCTACTCGTAGAGTAGACGACGCAACGGTG
101         A  N  P  T  T  A  E  T  L  D  A  T  R  R  V  D  D  A  T  V

361        GCCATAAGGAGCGCGATAAATAATTTAATAGTAGAATTGATCAGAGGAACCGGATCTTAT
121         A  I  R  S  A  I  N  N  L  I  V  E  L  I  R  G  T  G  S  Y

421        AATCGGAGCTCTTTCGAGAGCTCTTCTGGTTTGGTTTGGACCTCTGCCATGGACAGAGCA
141         N  R  S  S  F  E  S  S  S  G  L  V  W  T  S  A  M  D  R  A

481        CATTACAATATAGTTACTTTCGCCGGCCCTGCAACTTGA
161         H  Y  N  I  V  T  F  A  G  P  A  T  *
```

(4)

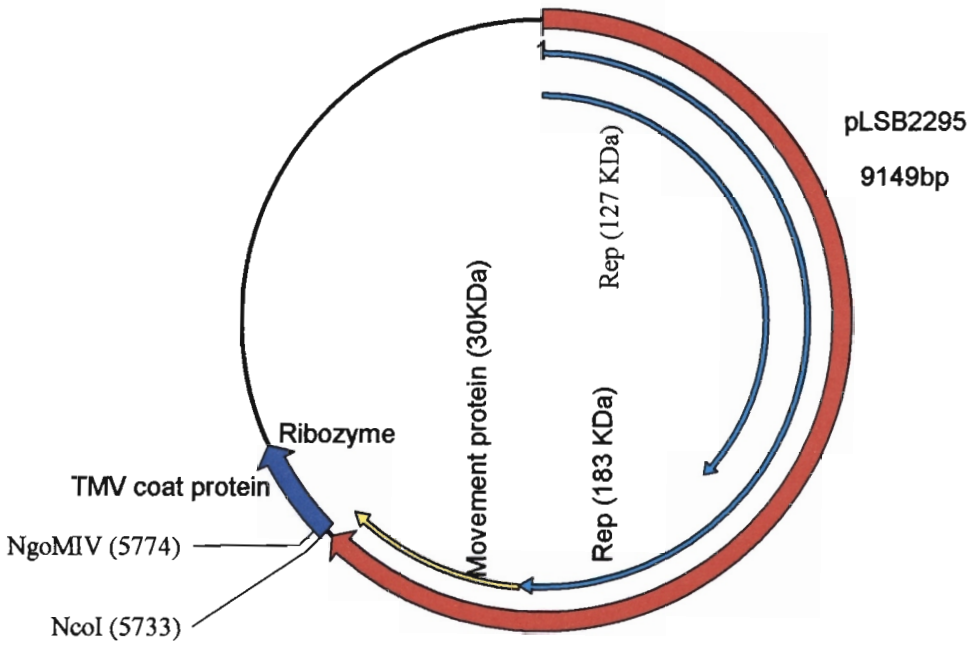


Figure B3 pLSB2295 TMV based vector provided by LSBC, which contains the NgoMIV/ NcoI cloning site at the N-terminus of the TMV coat protein.

(5) pLSB2295 coat protein sequence

Translation of pLSB2295 cp(1-486)
Total amino acid number: 161, MW=17639
Max ORF starts at AA pos 1(may be DNA pos 1) for 161 AA(483 bases), MW=17639

```
1       CCATGGNNGCCGGCTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCATCAGCG
1          M  X  A  G  S  Y  S  I  T  T  P  S  Q  F  V  F  L  S  S  A

61      TGGGCCGACCCAATAGAGTTAATTAATTTATGTACTAATGCCTTAGGAAATCAGTTTCAA
21         W  A  D  P  I  E  L  I  N  L  C  T  N  A  L  G  N  Q  F  Q

121     ACACAACAAGCTCGAACTGTCGTTCAAAGACAATTCAGTGAGGTGTGGAAACCTTCACCA
41         T  Q  Q  A  R  T  V  V  Q  R  Q  F  S  E  V  W  K  P  S  P

181     CAAGTAACTGTTAGGTTCCCTGACAGTGACTTTAAGGTGTACAGGTACAATGCGGTATTA
61         Q  V  T  V  R  F  P  D  S  D  F  K  V  Y  R  Y  N  A  V  L

241     GACCCGCTAGTCACAGCACTGTTAGGTGCATTCGACACTAGAAATAGAATAATAGAAGTT
81         D  P  L  V  T  A  L  L  G  A  F  D  T  R  N  R  I  I  E  V

301     GAAAATCAGGCGAACCCCACGACTGCCGAGACGTTAGATGCTACTCGTAGAGTAGACGAC
101        E  N  Q  A  N  P  T  T  A  E  T  L  D  A  T  R  R  V  D  D

361     GCAACGGTGGCCATAAGGAGCGCGATAAATAATTTAATAGTAGAATTGATCAGAGGAACC
121        A  T  V  A  I  R  S  A  I  N  N  L  I  V  E  L  I  R  G  T

421     GGATCTTATAATCGGAGCTCTTTCGAGAGCTCTTCTGGTTTGGTTTGGACCTCTCCTGCA
141        G  S  Y  N  R  S  S  F  E  S  S  S  G  L  V  W  T  S  P  A

481     ACTTGA
161        T  *
```

(6)

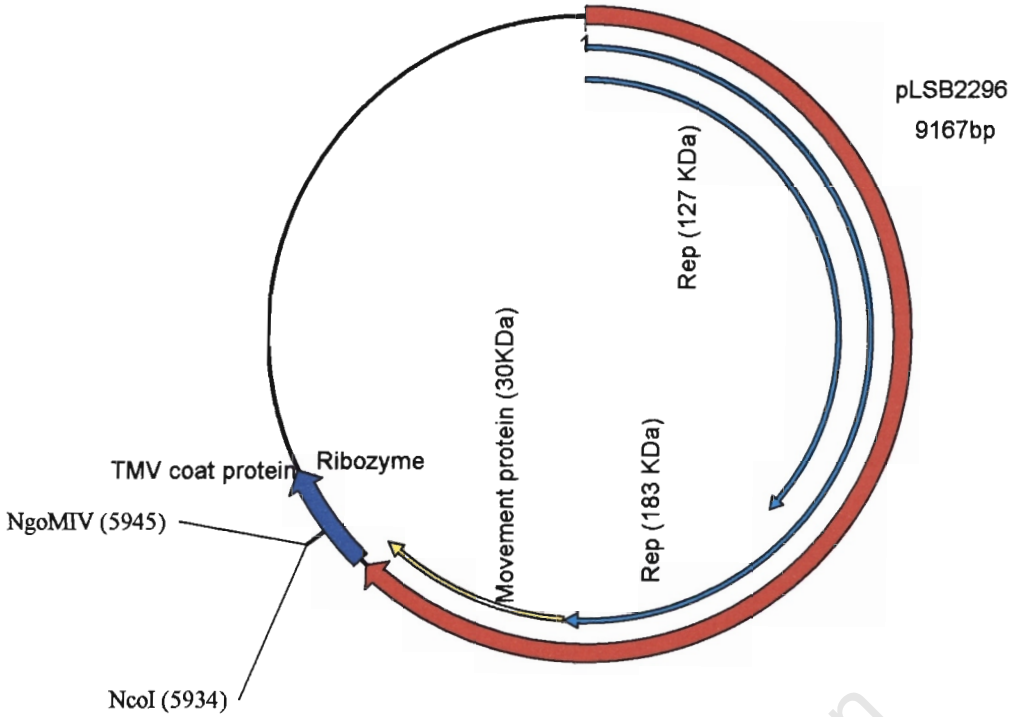


Figure B4 pLSB2296 TMV based vector provided by LSBC, which contains the NgoMIV/ NcoI cloning site at the 60'S loop region of the TMV coat protein.

(7) pLSB 2296 coat protein sequence

Translation of 2296 cp(1-504)
Total amino acid number: 167, MW=18210
Max ORF starts at AA pos 1(may be DNA pos 1) for 167 AA(501 bases), MW=18210

```
1          ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCATCAGCGTGGGCCGAC
1           M  S  Y  S  I  T  T  P  S  Q  F  V  F  L  S  S  A  W  A  D

61         CCAATAGAGTTAATTAATTTATGTACTAATGCCTTAGGAAATCAGTTTCAAACACAACAA
21          P  I  E  L  I  N  L  C  T  N  A  L  G  N  Q  F  Q  T  Q  Q

121        GCTCGAACTGTCGTTCAAAGACAATTCAGTGAGGTGTGGAAACCTTCACCACAAGTAACT
41          A  R  T  V  V  Q  R  Q  F  S  E  V  W  K  P  S  P  Q  V  T

181        GTTAGGTCCCCTGGATCTCCCATGGACAGTGCCGGCCCTTCTGGAGACTTTAAGGTGTAC
61          V  R  S  P  G  S  P  M  D  S  A  G  P  S  G  D  F  K  V  Y

241        AGGTACAATGCGGTATTAGACCCGCTAGTCACAGCACTGTTAGGTGCATTCGACACTAGA
81          R  Y  N  A  V  L  D  P  L  V  T  A  L  L  G  A  F  D  T  R

301        AATAGAATAATAGAAGTTGAAAATCAGGCGAACCCCACGACTGCCGAGACGTTAGATGCT
101         N  R  I  I  E  V  E  N  Q  A  N  P  T  T  A  E  T  L  D  A

361        ACTCGTAGAGTAGACGACGCAACGGTGGCCATAAGGAGCGCGATAAATAATTTAATAGTA
121         T  R  R  V  D  D  A  T  V  A  I  R  S  A  I  N  N  L  I  V

421        GAATTGATCAGAGGAACCGGATCTTATAATCGGAGCTCTTTCGAGAGCTCTTCTGGTTTG
141         E  L  I  R  G  T  G  S  Y  N  R  S  S  F  E  S  S  S  G  L

481        GTTTGGACCTCTCCTGCAACTTGA
161         V  W  T  S  P  A  T  *
```

All pLSB vectors contained a pUC backbone, which conferred ampicillin resistance. All pLSB vectors were prepared for ligation by *NcoI* and *NgoMIV* restriction enzyme digestion (Chapter 2.2.2) followed by Shrimp Alkaline Phosphatase treatment (Appendix A(III)) to prevent self-ligation and ensure that only vectors containing the annealed insert were capable of ligating.

APPENDIX C

PCR Assembly Components

I. Oligonucleotides used

Synthetic primer sequences used were synthesized by the Oligonucleotide Synthesizing Service of the Department of Molecular and Cell Biology, University of Cape Town, Cape Town, South Africa.

TableC1: Displaying Oligonucleotides used for the PCR assembly of the indicated V3-epitopes.

Primer	Du151a epitope	Du151b epitope	Du179a epitope	Du179b epitope
(A)	5′ACATGCCAT GGGTAACAACAACAC 3′	5′CATG CCATGGGTCG GAAGAGCATC 3′	5′CATG CCATGGGTGG CAACAACACC 3′	5′CATG CCATGGGTCG GAAGAGTATT 3′
(B)	5′CGATCCGGAT GCTCTTCCGG GTGTTGTTGT TACCCATGG 3′	5′GTCTGTCCTG GTCCTATTCG GATGCTCTTC CGACCCATGG 3′	5′CCAATTCGAA TACTCTTCCG GGTGTTGTTG CCACCCATGG 3′	5′GCCTGTCCTG GTCCAATTCG AATACTCTTC CGACCCATGG 3′
(C)	5′CCGGAAGAGC ATCCGGATCG GACCAGGACA AACATTTTAT 3′	5′CGAATAGGAC CAGGACAGAC ATTCTATGCA ACAGGAGAGA 3′	5′CGGAAGAGTA TTCGAATTGG ACCAGGACAG GCATTCTATA 3′	5′CGAATTGGAC CAGGACAGGC ATTCTATACA AACCACATCA 3′
(D)	5′AGCCGGCGGT TGCATAAAAT GTTTGTCCTG GTC 3′	5′AGCCGGCTAT TATCTCTCCT GTTGCATAGAAT 3′	5′AGCCGGCTGT ATAGAATGCC TGTCCTGGT 3′	5′AGCCGGCGAT GATGTGGTTT GTATAGAAT 3′

II. PCR temperature profiles

Stage 1 PCR profile:

94°C	2 min	
94°C	20 sec	X 10 cycles
40°C	20 sec	
72°C	15 sec	

Stage 2 PCR profile:

94°C	2 min	
94°C	20 sec	X 15 cycles
54°C	15 sec	
72°C	15 sec	

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