EXPRESSION OF HIV-1 SUBTYPE C NEF IN *E. COLI* AND *NICOTIANA BENTHAMIANA*; DEVELOPMENT OF PLANT-BASED VACCINES FOR HIV

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A thesis submitted to the Faculty of Science, Department of Molecular and Cell Biology, University of Cape Town, in fulfillment of the requirements for the degree of Master of Science

July 2003

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ACKNOWLEDGEMENTS

I wish to express my thanks to the following:

My ancestors without whom I would not be.

Peach, my pillar of strength in what promises to be an exciting and turbulent life.

My supervisor, **Prof Ed Rybicki**, for matching your high expectations with the necessary patience.

Dr Fiona Tanzer, from whom I learned so much about science and research, and a great deal more.

The South African AIDS Vaccine Initiative for their generous financial support.

The Postgraduate Scholarships Office for their equally generous financial support.

My research colleagues in the lab and in the SAAVI vaccine group for their support and contributions to my work.

Arvind, Eric, Livio and **Darren**, a great gang of mad doctors.

Pei Yin for oligonucleotide synthesis and **Di James** for sequencing services.

Jackie, Donald and **Blommie** for everything.

Mr G. P Bandawe and **Mrs A. T Bandawe**, my parents, for unrelenting support and faith in all my endeavous.

Terry and Felicia Van Graan who have been my parents in Cape Town.

Edward Theka for being a true friend.

ABSTRACT

Expression of the *nef* gene from HIV-1 subtype C in *Nicotiana benthamiana* was carried out using a TMV-based vector with the aim of developing a plant-based candidate vaccine for HIV-1. The *nef* gene of the DU151 isolate of HIV-1 subtype C taken from a recently seroconverted individual was amplified by PCR with a deletion of 59 amino acids from the cytotoxic N-terminal. The amplified gene was inserted into a bacterial expression vector pProEXHTb for rapid expression of Nef protein, which was used as a diagnostic tool in the development of an indirect ELISA assay for detection of Nef in *Nicotiana benthamiana*. An indirect ELISA assay was developed using a commercially available polyclonal anti Nef antiserum raised in sheep.

The role of codon optimization in expression of Nef in *benthamiana* was investigated. A synthetic *nef* gene was constructed based on the codon usage of *benthamiana*. The plant codon optimized gene and the wild type *nef* genes were inserted into the TMV-based vector pBSG1057. RNA transcripts from both constructs were used to infect young *benthamiana* plants. Expression of *nef* mRNA was confirmed by RT-PCR analysis of total RNA extracted from plants inoculated with respective constructs. The Nef protein was expressed at low levels which were detectable by ELISA. Nef was detectable by Western blot after concentration of plant extract using a membrane filter device. Quantitative analysis of Nef expression in plants was done by western blot on concentrated plant extract from three separate infections. Codon optimization of the *nef* gene improved the expression of Nef by a factor of about two.

Expression of immunodominant CTL epitopes from the core region of Nef as TMV-CP translational fusions was attempted. Oligonucleotides encoding the peptides YKAAFDLSHFLKEKG (NP31) and EEPEVGFPV (EP1) were synthesized and cloned into the vector pBSG1057 in frame with the coat protein gene using an intermediate vector to be expressed as N- and C-terminal fusion products. No infectious rTMVs were generated by plants inoculated with RNA transcripts of these constructs and infections remained localised. RT-PCR of total RNA extracted from inoculated plants revealed the presence of mRNA encoding rTMV-CP. Western analysis of plant extracts showed no accumulation of TMV-CP in plants infected with NP31 constructs and poor accumulation of TMV-CP in plants infected with EP1 constructs. Both peptides interfered with expression and assembly of TMV particles.

ABBREVIATIONS

AIDS	acqired immune deficiemcy syndrome
AlMV	alfalfa mosaic virus
bp	base pair
cDNA	complimentary deoxyribonucleic acid
CP	capsid protein
CPMV	cowpea mosaic virus
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
DWR	dynamic working range
ELISA	enzyme linked immunosorbent assay
FMDV	foot and mouth disease virus
g	gram
GFP	green flourescent protein
HAART	highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HPV	Human Papilloma virus
IMAC	immobilised metal affinity chromatography
IgA	immunoglobulin A
kb	kilobase
kDa	kilo Dalton

LA	Luria-Bertania agar
LB	Luria-Bertania broth
LSB	Large Scale Biology Corporation
mg	milligram
MHC	major histocompatability complex
mRNA	messenger RNA
ml	millilitre
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVX	potato virus X
rBV	recombinant baculovirus
RNA	ribonucleic acid
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SIV	simian immunodeficiency virus
SHIV	chimeric SIV/HIV virus
TMV	tobacco mosaic virus
UTR	untranslated region
VLP	virus-like particle

CHAPTER 1

Introduction and Literature Review

1.1 HIV/AIDS

1.1.1 Epidemiology

Since AIDS was recognized as a new disease in the beginning of the 1980s and HIV was isolated and identified as a transmissible pathogen causing this disease, HIV has spread all over the world and has already claimed more than 25 million lives. The number of persons currently living with HIV infection worldwide is estimated by the Joint United Nations Program on HIV/AIDS (UNAIDS) at more than 40 million. About 5 million new infections with HIV occurred in the year 2001 and about 3 million people died from HIV/AIDS, making HIV infection the single most important cause of death from infectious disease in that year (www.unaids.org). More than 95% of the approximately 15000 new infections per day are transmitted in developing countries. The most severely affected part of the world is sub-Saharan Africa, with more than two thirds of all infected persons living in this region and 3.4 million new infections occurring in 2001. More Africans die from HIV/AIDS than from famine and wars and there are now 16 countries where more than 10% of those aged 15-49 years are infected. In South Africa's Kwazulu-Natal province, prevalence rates among women attending antenatal clinics have reached catastrophic proportions with the figure standing at 36% (Department of Health, Republic of South Africa 2000).

The principal mode of transmission of HIV in Africa is unprotected heterosexual intercourse which is responsible for more than 85% of new infections. About 10% of new infections are due to mother-to-child transmission. Intravenous drug use has not played a large role in dissemination of HIV in Africa but recent studies show that use of non-sterile surgical equipment in medical and cultural practices may play a significant role (UNAIDS).

1.1.2 Molecular Biology of HIV

HIV belongs to the subfamily *Lentivirinae* (slow viruses) in the family Retroviridae. In general, retroviruses either cause proliferation (transforming) or destruction (cytopathic) of the cells they infect. Human T-cell leukaemia viruses HTLV-1 and HTLV-2 are examples of transforming viruses. HIV is a cytopathic virus. There are two HIVs: HIV-1

and HIV-2. They share about 30% to 40% RNA sequence homology overall, with as high as 60% homology in some regions. HIV-1 is by far the most common cause of AIDS. HIV-2 produces a disease similar to HIV-1 and is present in high incident in West Africa but is being found increasingly outside Africa. HIV-2 is very closely related to simian immunodeficiency viruses (SIV), which cause a form of AIDS with encephalitis in monkeys (macaques) that is very similar to AIDS in humans (SIVmac) (Fultz *et al.*, 1989).

The life cycle of HIV can be briefly summarised as follows. The mature virion forms a sphere with 72 spikes that contain the Env (gp120 and gp41) glycoproteins. A region of the viral envelope gp120 binds to a domain on the CD4 protein on the lymphocyte surface. This interaction causes a conformational change in the gp120, resulting in co receptor binding. Although several chemokine receptors such as CCR1, CCR2b and CCR3 can function as coreceptors for cell entry, CCR5 and CXCR4 are the most relevant (Rucker *et al.*, 1997). The viral envelope complex that interacts with the CD4 and chemokine cell membrane receptors is composed of six glycoprotein subunits: three gp120s and three gp41s (Trkola *et al.*, 1996). Binding of the HIV gp120 to CD4 leads to a conformational change in the envelope that creates a high affinity binding site for the chemokine receptor (Trkola *et al.*, 1996). Interaction of the gp120, CD4 and the chemokine receptor causes structural changes that allow the external portion of gp41 (the fusion domain) to mediate the fusion of the viral and cell membranes. The fusion of the viral and cell membranes creates a pore which permits the HIV capsid to pass into the CD4 cell, releasing the viral RNA into the cell cytoplasm (Lapham *et al.*, 1996). Once the virion is uncoated in the cytoplasm, virion-associated reverse transcriptase produces hybrid RNA/DNA molecules. These are converted to double stranded cDNA molecules. The linear HIV cDNA is translocated to the nucleus of the infected cell where it is integrated into the host DNA by the viral integrase enzyme (Burger and Poles, 2003).

In the integrated proviral form HIV may remain latent in infected cells for months or years. The HIV genome consists of nine viral genes that are flanked by long terminal repeats (LTRs). These genes include the major *gag* gene, which encodes the viral capsid

structural protein; *pol*, which encodes enzymes required forintegration and replication as well as the viral protease protein; and *env*, which encodes the envelope glycoproteins. Low level transcription of the viral genome results in the production of multiply spliced RNA transcripts and the translation of the *tat* and *rev* early genes products. Tat functions as a transactivator, amplifying transcription of additional viral RNA transcripts, and Rev enhances export of viral RNA to the cytoplasm from the nucleus, where they are translated into proteins. On translation of the late Gag, Pol and Env viral proteins, virion assembly occurs at the cellular plasma membrane with two copies of single stranded genomic HIV RNA in each progeny virion. During budding from the cell, surface proteins such as MHC molecules are also incorporated into the viral coat. The final maturation steps, such as cleavage of the Gag and Pol precursor proteins and other post-translational modifications of viral proteins, occur during and after the budding process. Acquisition of host proteins in the viral coat proteins produces "pseudotypes" of HIV with different host-derived surface proteins (Burger and Poles, 2003).

1.1.3 Treatment

To date, about 15 different antiretroviral drugs have been approved for use with HIV infections and are in regular use. Current antiretroviral therapy nearly always involves a combination of three or more drugs, which need to be given at regular intervals. A typical triple combination therapy (HAART) regimen includes either two inhibitors of the HIV protein reverse transcriptase (RT) (nucleoside RT inhibitors as well as non-nucleoside RT inhibitors) plus one inhibitor of the viral protease or a combination of three different RT inhibitors (usually 2 nucleoside RT inhibitors and 1 non-nucleoside RT inhibitor) (Marcus *et al.*, 2002). Under HAART, significant suppression of virus load, usually to undetectable levels, can be achieved in most patients. Patient care has been transformed with the ability to withdraw prophylactic or suppressive chemotherapy for pneumocystis, taxoplasmosis, cytomegalovirus, *Mycobacterium avium* complex, leishmaniasis, cyptococcosis and candidal thrush, which previously had been lifelong afflictions (Kovacs and Masur, 2000). However, significant side effects, including anaemia, neutrophia, allergic reactions and lipodystrophy are just some of the challenges facing successful implementation of HAART. Because of problems with adherence,

pharmacology and toxicity, only 50-90% of study subjects achieve the desired suppression with current regimens (Richman, 2000). More significant is the alarming emergence of drug resistance. There are increasing reports in the literature of transmission of resistant virus. Such documentation has expanded from anecdotes of transmission of AZT-resistant virus to the identification of large cohorts with 10% or more of new infections due to drug resistant virus, half of which exhibit resistance to multiple classes of drug (Little, 2000). This problem is anticipated to cause a significant loss of the therapeutic arsenal in the near future (Marcus *et al.* 2000).

The benefits of improved treatment so far have been restricted to only a minority of patients, mainly from the developed countries. Even though these therapies are cost effective by western standards at US$10,000-15,000 per year of life saved, most developing countries cannot afford the drugs as well as the necessary monitoring and support (Forsythe, 1998). In South Africa widespread provision of antiretroviral therapy is yet to be achieved and has become a major political issue.

The ultimate shortcoming of current therapies is that even in the healthiest and longest surviving HIV infected individuals, examination of tissues and blood for viral RNA and for short half-lifed closed circular reverse transcripts, as well as documentation of nucleotide sequence evolution, all indicate that most effectively treated patients are not completely suppressing virus replication but instead are experiencing intermittent or smouldering virus replication that is difficult to discern (Fischer *et al.,* 2000). This incomplete suppression of viral replication, which is usually on a scale sufficient to exert selective pressure, drives the evolution and fixation of drug resistant virus at an unprecedented rate contributing to the worldwide incidence of drug resistance (Richman, 2001)

1.2 VACCINE-BASED STRATEGIES FOR HIV PREVENTION

1.2.1 Subunit vaccines

Vaccination is one of the most cost effective and important methods of preventing infectious diseases. Owing to the worldwide vaccination programs, the incidence of many fatal diseases has drastically decreased. Most of the vaccines used routinely today as part of childhood immunization programs are whole organism vaccines, consisting of live attenuated or killed whole bacteria or viruses (Plotkin, 1993). However, there exists the risk of reversion to virulent wild-type strains that can lead to disease when using attenuated bacteria or virus, especially in immunocompromised hosts. In 1986, the first recombinant subunit vaccine, the hepatitis B surface antigen vaccine produced in *Saccharomyces cerevisiae* (*S. cerevisiae*) (Valenzuela *et al.*, 1982), was licensed. The basic principle for subunit vaccines is that the gene encoding the subunit vaccine is isolated transferred to a second, normally non-pathogenic organism. The recombinant subunit vaccine is then produced by the heterologous host, and could be designated to be delivered either as a purified immunogen, or by using the production host as live vector. The advantages of subunit vaccines are numerous. Firstly, the pathogen can be entirely excluded from the production of the vaccine, which eliminates risks associated with production, as well as risks of contamination with toxic compounds, risks of reversion to virulent genotypes or incomplete inactivation of whole-cell vaccines. By optimizing the delivery system, the immune responses can be tailored for specific pathogen against which the vaccination is aimed, and many recombinant subunit vaccines are investigated for mucosal delivery, using appropriate vectors.

Two immunological approaches have evolved for the development of anti-HIV vaccines: prophylactic and therapeutic vaccines. The prophylactic vaccines would elicit a humoral immune response since they induce the production of antibodies that are capable of neutralizing the viral antigen before it enters the cell and hence the target of choice would be the envelope proteins. Therapeutic vaccines are intended to induce cellular components of the immune system to recognize and attack HIV infected cells and hence viral accessory genes are considered the appropriate therapeutic agents since they are expressed through the various stages of HIV infection.

1.2.2 Prophylactic vaccines

A prophylactic vaccine would aim to induce a response that causes neutralization of the free virus particles by antibodies. This activity is considered the most important for antibody-mediated protection. It is defined as "the loss of infectivity which ensues when antibody molecules bind to a virus particle, and usually occurs without the involvement of any other agency. As such it is an unusual activity of antibody paralleled only by the inhibition of toxins and enzymes". In order to achieve protection through these means, a vaccine should aim to elicit antibodies of the highest affinity for virion surface antigen (Burton, 2002). For this reason, early vaccine candidates concentrated on eliciting responses to Env. However, with these early vaccine candidates several important factors became apparent. Because genetic variability is one of the hallmarks of HIV, neutralizing antibodies, which are predominantly directed against the V3 loop of the envelope protein (gp120), react only with a small number of virus isolates. Other antibodies, especially those directed against conformational epitopes of the CD4 ligand of gp120 or transmembrane protein gp41, can neutralize a wider range of HIV-1 isolates. However, these antibodies are rarely, if ever, induced by experimental vaccination. Another extremely important consideration was that because the usual route of transmission of HIV-1 is through mucosal surfaces, mucosal immunity may be required in order to prevent sexual transmission of the virus. Two studies have investigated the ability of antibodies to protect against infection from intravenous challenge (Mascola *et al.*, 1999) and through mucosal surfaces, with encouraging results. Using chimaeric SIV/HIV viruses (SHIVs) in non-human primates, Mascola and colleagues infused neutralizing antibodies into rhesus macaques to protect against vaginally transmitted infection (Mascola *et al.*, 2000), while Baba and co-workers tested the effects of such antibodies in an oral mucosal exposure after birth (Baba *et al.*, 2000). Passive transfer of antibodies conferred protection against disease in both studies. Although relatively high concentrations of antibody (>100ug ml^{-1}) were used—levels much higher than would ordinarily be achieved by vaccination—these studies showed that an appropriate serum antibody response might reduce infection at mucosal surfaces. It is unlikely that vaccination could achieve such a robust antibody response, although vaccines could also generate cellular immunity that might reduce the requisite neutralizing antibody

concentration to protect against infection. It is also hoped that synergy between antibodies directed against different neutralizing determinants might reduce the concentration required for effective neutralization (Nabel, 2001).

Thus far, attempts to design vaccines that stimulate neutralizing antibodies have been disappointing. No vaccine tested to date in monkeys with SIV or SHIV models has induced a sterilizing immunity, and all animals subsequently challenged became infected (Marcus *et al.*, 2002). Results from the long-awaited phase 3 trials of gp120 (AIDSVAX) reported in February 2003, have also been very disappointing, with these having been shown to be completely ineffective (VaxGen Inc Press release 24 February 2003).

1.2.3 Therapeutic vaccination

Therapeutic vaccination is an alternative approach to HIV treatment that has been investigated by a number of groups in the last decade. The main objective has been to augment virus-specific host immune responses, both humoral and cellular, believed to be critical in achieving control of viral replication (Lisziewicz *et al.*, 2003). Three main types of therapeutic vaccines have been clinically tested to date. Firstly, subunit vaccines containing recombinant env proteins (rgp160/rgp120) have advanced in clinical trials (reviewed in Lisziewicz *et al.*, 2003), reflecting the preventive vaccine effort based on the fact that neutralising antibodies are directed primarily against these proteins. Subunit vaccines of HIV-1 immunogen (whole inactivated HIV, depleted of gp160 and gp120 proteins) and other viral proteins have also been developed (reviewed in Lisziewicz *et al.*, 2003). Secondly, recombinant viral vectors encoding viral proteins are able to carry large sequences of DNA, and by mimicking microbial infection, induce both humoral and cellular responses to these antigens in vivo (Jin *et al.*, 2002). DNA vaccines have been tested with the first clinical trial in HIV infected individuals reported in 1998 (MacGregor *et al.*, 1998).

Clinical trials of antiretroviral drugs have employed viral load as the surrogate marker to detect clinical efficacy. For the testing of therapeutic vaccines no immunological end-point has been identified which correlates to survival in a similar manner. All of the

experimental therapeutic vaccines investigated have been safe and well tolerated. Many have demonstrated immunogenicity, as measured with a variety of immunological parameters, but none have yet demonstrated clinical benefit (Lisziewicz *et al.*, 2003)

1.3 EXPRESSION SYSTEMS FOR VACCINES

1.3.1 Recombinant subunit vaccine production hosts

A large number of different hosts are being investigated for recombinant antigen production. Commonly used systems for recombinant protein production and their characteristics are listed in table 1.1. Each host offers its own advantage, but there are also limitations, which have to be considered when choosing the host.

TABLE 1.1: Comparison of various systems for expression of foreign proteins

	Growth media	Equipment costs	Level of protein production	Purification	Sensitivity to growth conditions *	Posttranslational Processing #	Codon usage#
Bacteria	Moderate	Moderate	High	Easy to moderate	Moderate	Poor	Poor
Insect cells	Moderate to high	Moderate	Moderate to high	Easy to moderate	Moderate	Moderate	Good
Animal cells	High	High	Low	Moderate to difficult	High	Excellent	Excellent
Yeast cells	Moderate	Moderate	High	Easy to Moderate	Moderate	Moderate to excellent	Poor
Plants	Low	Low	Low	Moderate to difficult	Low	Moderate	Moderate

*Shear forces, pH, temperature, oxygen; #expression of mammalian proteins (Awram *et al.*, 2002)

1.3.1.1 Expression in *E. coli*

The advantage of using *E. coli* for heterologous protein production is its ability to be grown rapidly at high density on inexpensive substrates, its well characterized genetics and the availability of a large number of cloning vectors (Baneyx, 1999). The main concern with using *E. coli* is that overproduction of heterologous proteins in the cytoplasm of *E. coli* is often accompanied by their misfolding and segregation into insoluble aggregates known as inclusion bodies. Although inclusion body formation can greatly simplify purification, there is no guarantee that the *in vitro* refolding will yield

large amounts of biologically active products. The arginine codons AGA and AGG are rarely found in *E. coli* genes; the presence of such codons in cloned genes affects protein accumulation levels and mRNA and plasmid stability, in extreme cases it inhibits cell growth and hence protein synthesis (Zahn, 1996).

1.3.1.2 Recombinant baculovirus as expression vectors for insect cells

Baculovirus expression vectors continue to be used extensively for the expression of a variety of recombinant proteins in insect cells. These include cytosolic, nuclear, mitochondrial, membrane-bound and secreted proteins (Kost and Condreay, 1999). Recombinant baculovirus (rBV) systems are being increasingly used for the development of subunit vaccines. Co-expression of viral proteins has been used to achieve higher yields and stability of the particles (Kirnbauer *et al.*, 1993; Xi and Banks, 1991). Recombinant BVs have been extensively used to express a variety of viral proteins including the HIV-1 gag protein (Nermut *et al.*, 1994). Despite the high level of expression of recombinant proteins, the baculovirus system is relatively expensive. Baculovirus expression systems provide a great tool nonetheless for the functional and antigenic properties of rBV expressed proteins.

1.3.1.3 Expression in *S. cerevisiae*

The advantage of yeasts as an expression system is that like bacterial systems they are easy to culture on inexpensive media and there is a formidable array of techniques for the manipulation of foreign genes. Since they provide an environment for post-translational processing and secretion the result is a product that is often identical or very similar to the native protein (Sudbery, 1996). The yeast *S. cerevisiae* has been widely used for the production of VLPs of many different viruses. Antigens of interest expressed in *S. cerevisiae* include HIV-1 Gag protein (Sakuragi *et al.*, 2002; Jacobs *et al.*, 1989) anti-malarial antigens (Brady *et al.*, 2001), polio virus subviral proteins (Rombaut and Jore, 1997) and hepatitis B surface virus antigen (Valenzuela *et al.*, 1982). Due to its application in the bread and alcoholic beverages, industrial scale fermentation facilities are already in place for large-scale production and sterility may not be of critical importance.

1.3.2 Options for low-cost vaccines for developing countries

A vaccine strategy employing purified virus-like particle (VLP) preparations is too costly for widespread vaccination to be implemented in developing countries, where the highest incidence of HIV occurs. Strategies applicable to these settings are thus under investigation. The requirement for delivering HIV proteins in appropriately immunogenic form remains a problem, as well as additional features, such as low cost, stability and single dose efficacy. Live vectors for delivery of heterologous subunit antigens offer a number of advantages as a vaccination strategy. Both Gram-negative and Gram-positive bacteria, including *Mycobacterium* species, as well as a whole range of viruses, have been investigated for delivery of foreign antigens. These live recombinant bacterial and viral vectors are able to replicate and express heterologous antigens *in vivo*. Cell-mediated and antibody responses may be elicited, and these vectors may be suitable as therapeutic and prophylactic vaccines.

1.3.2.1 Live viral vectors

Genes encoding relevant antigens can be spliced into recombinant expression vectors allowing for increased cellular production of the antigen and induction of humoral immune and cellular responses (Mackett *et al.*, 1982). Viruses that can be used include vaccinia virus, alphaviruses and adenovirus. The advantages of vaccinia virus systems are the wide host range of mammalian cells that can be infected, the high expression levels, the ease of virus stock production and the fact that it remains the most successful and possibly best understood vaccine. A disadvantage of the system is that it requires bio safety level 2 for production. The vaccinia virus system has been successfully used for large scale (1000L) production of different proteins such as HIV-1 rgp160 by Pasteur-Merieux and human pro-thrombin by Immuno AG (Wurm and Bernard, 1999).

Sindbis virus or Semliki Forest virus (SFV) are the most studied alphaviruses used for heterologous expression (Lundstrom, 1997). The broad host range of SFV has made it particularly attractive for production of recombinant proteins since the extremely efficient SFV 26S subgenomic RNA promoter used in these SFV expression vectors, comprising of two plasmids (cloning vector pSFV1 the carries the replicase genes and pSFV2-helper

plasmid which encodes the structural proteins of the virus) and the high number of copies of RNA (200 000) per infected cell, leads to high expression. Scale up for alphaviruses has been established for SFV but this unfortunately requires high biosafety level production facilities (Lundstrom, 1997).

Adenoviral vectors are not pathogenic in humans, can be made replication competent or deficient, and can be administered orally (Imler, 1995). Humoral, cell-mediated and mucosal immunity can be elicited to the heterologous antigens delivered by adenoviruses (liljeqvist and Stahl, 1999). Several viral antigens such as HBV surface antigen, the measles virus nucleocapsid and glycoproteins from herpes simplex virus and rabies virus have been expressed and delivered by adenoviral vectors. Recombinant polioviruses have used to express a variety of viral antigens including HBV surface antigen and SIV proteins (Tang *et al.*, 1997; Yim *et al.*, 1996; Crotty *et al.*, 2001).

Despite the numerous studies, including clinical studies, conducted involving live recombinant viral vectors performed, no such vaccine candidate has progressed past phase II evaluation, since profiles of the immune response elicited were not considered ideal. Liljeqvist *et al.*, (1999) speculate that until the safety and immunological questions are solved, only certain specific vaccines such as HIV and cancer in addition to veterinary vaccines, would be the applications for virus-based vectors.

1.3.2.2 Live bacterial vectors

Live bacterial vaccines are relatively inexpensive to manufacture and are well suited to large-scale administration in both developed and developing countries. They have the added advantage that they can be administered orally, which is practical and reliable in large-scale vaccination programs (Shata *et al.*, 2000). All field and clinical trials with *Salmonella* as a delivery system have been limited to attenuated *Salmonella typhi*, however a promising alternative as a vaccine vector is *S. typhimurium* for heterologous antigen delivery (Nardelli *et al.*, 1997).

Bacille Calmette-Guerin (BCG) as vaccine vector has the advantage of the established safety in humans and its capacity to accommodate large fragments of foreign DNA (Lugosi *et al.*, 1989; Jacobs *et al.*, 1990). Current information suggests that rBCG vaccines may be beneficial to prime or retain memory responses to antigens, but are unlikely to be useful as a single component vaccine strategy.

1.3.2.3 DNA vaccines

DNA vaccines have emerged as an attractive vaccine strategy because they are stable and relatively cheap, quick and easy to produce. The DNA can be administered by a variety of routes, all of which result in DNA uptake by antigen-presenting cells (APCs) and other cells as well as expression of the DNA-encoded antigen (Steller, 2002). Expression of foreign proteins within host cells from naked DNA constructs allows for the induction of humoral and cell-mediated responses, whilst protein subunit vaccines may only induce antibodies (Ulmer *et al.*, 1996a; Ulmer *et al.*, 1996b). DNA can be propagated as plasmids in bacteria and purified with ease and at a low cost. Chen *et al.,* (2002) found that priming with DNA and boosting with a vaccinia virus-expressed subunit vaccine generated a stronger immune response than either of the two on their own. Immunization with DNA vaccines encoding Nef, Rev and Tat induced lymphoproliferation, cytotoxic T-lymphocyte (CTL) and antibody responses in asymptomatic HIV-1 infected patients (Calarota *et al*, 1999)

1.3.2.5 Cell-based vaccines

Preclinical models have shown that administration of peptide with an appropriate adjuvant is important for enhancing the immunogenic stimulus (Vitiello *et al.*, 1995). Dendritic cells (DCs) have a critical role in *in vivo* antigen presentation and could as act as a useful adjuvant (Steller *et al.*, 2002). DCs are significantly more efficient in inducing anti-tumor protection than immunization with peptides alone (Celluzzi *et al.*, 1996) (Paglia *et al.*, 1996).

1.4 PLANT-BASED VACCINES

Interest in vaccine production in plants has expanded rapidly since there are considerable advantages in expressing antigenic proteins in plants. Posttranslational protein processing is comparable to mammalian cells (Bosch *et al.* 1994), allowing the expression of functionally active proteins including complex molecules such as antibodies (Hiatt *et al.* 1989). Plants can be growth locally and cheaply using standard non-sterile methods, thus reducing the issues with distribution, storage and transport. The use of plants as bioreactors is of interest as they allow the production of large quantities of recombinant proteins at relatively low cost. The whole production is flexible and easily changed to market needs (Herbers and Sonnewald, 1999). The cultivation, harvesting, storage, and processing of a transgenic crop would also use an existing infrastructure and require relatively little capital investment (Ganz *et al.*, 1996; Pen, 1996; Whitelam, 1995). Kusnandi *et al.* (1997) have estimated that the cost of producing recombinant proteins in plants could be 10- to 50- fold lower than in *E. coli*. Plant derived products, whether purified or not, are less likely to be contaminated with human pathogenic microorganisms than those derived from mammalian cell cultures since plants do not harbour human infectious pathogens, therefore sterility is not a big concern.

1.4.1 Stable transgenic expression systems

There are two main systems for expression of foreign proteins in plants; these are transgenic and transient. In the first, stably transformed transgenic plants are produced using *Agrobacterium*-mediated transformation, particle bombardment or other standard transformation techniques. *Nicotiana tabacum* is widely used as a model expression system, but various other suitable plants including *Nicotiana benthamiana*, *Arabidopsis thaliana*, tomato, banana, oilseed rape, Ethiopian mustard, lettuce, rice, wheat and maize have been used. *Agrobacterium* is a plant pathogen that can transform a large range of hosts including plants, fungi and animal cells. *Agrobacterium*-mediated transformation allows the integration of a gene cassette (T-DNA) into the plant genome. The process requires incubation of plant tissue with a bacterial culture containing the recombinant T-DNA. Plant tissue culture is then used to select for the recombinant cells, usually using an antibiotic resistance marker. The resulting transformed tissue is then regenerated into

plants. The time required for this process varies from 6 weeks to over 1 year depending on the plant species involved. Once a transgenic plant is obtained, the integration is stable and can be passed on by propagation using normal methods (i.e., seed and cuttings) (reviewed in Newell *et al.*, 2000). Expression levels in transgenic plants can vary considerably due to several reasons. Integration of the gene is essentially random and subsequent protein expression can be affected by copy number, positional effects and gene silencing. It is therefore necessary to screen a number of regenerated plants to determine expression levels. It is also necessary to select for plants that express stably over several generations.

Exceptionally high levels of protein have been achieved by integrating genes into the chloroplast genome where the recombinant protein can represent as much as 46% of the total soluble protein (De Costa *et al.*, 2001). Each plant contains thousands of chloroplasts, resulting in thousands of copies of the transgene that can be expressed. Positional and silencing effects are avoided because DNA is integrated by homologous recombination. Polycistrons can be expressed in the chloroplast allowing expression of multiple proteins and because chloroplasts have fewer proteolytic pathways than the remainder of the cell, foreign proteins may not be subjected to as much degradation when expressed in chloroplasts (Bock, 2001). In addition to high level expression, chloroplast integration should reduce the potential for transgene spread via pollen because chloroplasts are maternally inherited in most plants (Awram *et al.*, 2002). However, chloroplasts are essentially prokaryotes so the same limitations to producing eukaryotic proteins in such systems apply.

Although transgenic technologies have been used for the successful production of a number of experimental plant based vaccines, the time required to produce transgenic plants represents a major disadvantage, particularly if many different expression cassettes or promoter elements require evaluation. Therefore as noted below, a number of researchers have sought the more rapid expression systems offered by the use of plant virus expression vectors.

1.4.2 Plant virus expression systems

1.4.2.1 Advantages over transgenic expression systems

The second strategy is to infect non-transgenic plants with recombinant plant viruses that express transgenes during their replication in the host. The general approach adopted is to insert the foreign gene into the viral genome under the control of a strong subgenomic promoter. The resulting recombinant viruses can then be introduced into the appropriate host plant by mechanical inoculation, where after the virus can spread systemically throughout the plant. If the construct allows viable virus particles to be produced, the recombinant virus has the potential to spread from plant to plant. This property makes propagation simpler but may create containment issues. With few exceptions, most plant viruses do not integrate into the host cell genome. This property gives transient expression systems the advantages that foreign genes are not generally heritable through seeds and expression does not suffer from the positional effects that affect gene expression levels found in stably transformed transgenic plants (Awram *et al.*, 2002). Furthermore, transient expression using viral vectors is a fast and relatively simple method for examining proteins for desired characteristics since the infected plants can produce high amounts of proteins within 1-4 weeks of inoculation. This method generally requires less investment in time compared to transgenic plants before an expressed protein can be examined.

1.4.2.2 Problems affecting plant virus expression systems

There are, however a number of difficulties involved with the use of plant virus vectors. Inoculation of plants can be inefficient and expensive. Most viral vectors are RNA viruses and inoculation of the plant is accomplished by mechanical inoculation of the infectious RNA transcripts onto leaves. These transcripts are susceptible to rapid degradation from nucleases and are preferably used quickly after production. Systemic spread of the virus from the point of inoculation is also required and it has been found that some constructs interfere with the subsequent movement of the virus from the initial infectious centre (Cruz *et al.*, 1996).

The major problem associated with the use of plant viral vectors is genetic instability caused by the presence of the inserted foreign gene. Because the foreign gene is not required for the viral life cycle, and because multiple consecutive replication steps are involved in the process of systemic infection of a plant, the insert is commonly deleted or modified during infection. Some researchers have also found that certain vectors are unstable in E. coli plasmids making it difficult to maintain the infectious full length molecule that contains the intact foreign gene. Despite these difficulties, plant viral vectors exhibit significant advantages over transgenic plants for the production of foreign proteins. The short period of time required and the ability to infect grown plants with minimal manipulation allows for rapid analysis of many different constructs. The fact that these viruses can infect a range of different plant species means that the same construct can be used in several different plants to examine species effects on protein production and a number of different plant virus systems have been developed for protein expression (Awram *et al.*, 2002).

The host virus systems frequently used are cowpeas with cowpea mosaic virus (CPMV), tobacco with potato virus X (PVX), alfalfa mosaic virus (AIMV) and tobacco mosaic virus (TMV) (table 1.2). The product yields are generally higher than those from transgenic plants. In addition to expressing the entire coding sequence of a protein using a duplicated subgenomic promoter, another approach of transient expression is making chimaeric plant viruses that display epitopes / peptides on the surface of the virus capsid. This approach involves engineering of the virus coat protein to express the antigenic peptide as a translational fusion. Such recombinant coat protein monomers have the potential to self assemble and form recombinant virus particles displaying the desired antigenic epitopes on their surface. Because the coat protein accumulates to high concentrations and viruses can easily be isolated, this approach tends to result in high yields of heterologous proteins. Constructs that use native or heterologous coat proteins and subgenomic promoters, have enabled foreign proteins to be expressed to levels of 2% of the total soluble protein (Shivprasad *et al.*, 1999).

1.4.3 Enhancement of protein yield

1.4.3.1 Factors limiting yield

A major limiting factor in the use of plants to express proteins is often the low yield of recombinant protein that is achieved. An expression level of 1% of total soluble protein is generally accepted as a minimal level for it to be economically feasible or practicable to purify protein from a genetically modified plant (Kusanadi *et al.*, 1997). As a result, a number of methods have been developed to increase expression levels. These methods have been applied to transgenic expression and viral expression, although some methods may not work with certain viral systems.

Expression can be affected by factors on several different levels. At a transcriptional level, the use of appropriate promoters, enhancers and leader sequences for the expressed protein must be determined. At a translational level appropriate codon usage and removal of mRNA destabilizing sequences and polyadenylation signals from the foreign gene need to be addressed to enhance mRNA stability. And finally protein stability is crucial for accumulation of foreign proteins at high levels. These factors in turn depend on the plant system chosen and the method of expression as well as the stability of the protein. Some methods used in enhacement of expression are explained below.

1.4.3.2 3' mRNA stabilizing sequences

One of the most effective methods of increasing protein yields has proven to be the addition of 3' mRNA-stabilizing sequences. Richter *et al.* (2000) found that swapping the 3' region from nopaline synthatse with the 3' regions from either the soybean vspB gene or the potato pinII gene resulted in increases in RNA levels that translated into 20 to 50-fold increases in expression of hepatitis B surface antigen in potatoes.

1.4.3.3 5'UTRs

Another effective means of increasing protein production is by modification of leader or 5' untranslated regions (UTRs). The sequence of the UTRs greatly affects the levels of protein synthesis by altering ribosome binding and the initiation of protein synthesis (Beachy, 1997). It is not well understood what features of the UTR cause the differences

in protein synthesis. The best-studied UTRs are of viral origin, but much work has also been done on characterizing the UTRs of chloroplast mRNAs (Cohen and Mayfield, 1997).

1.4.3.4 RNA stability and codon usage

Plants may incorrectly recognize some sequences from foreign genes as mRNA-destabilising sequences and polyadenylation signals, which will also reduce yields. Such sequences result in lower production of mRNA and reduce the half-life of the mRNA molecule (Awram *et al*, 2002). In addition codon usage in plants may differ considerably from foreign genes derived from other organisms such as animal pathogens. This may result in reduced protein expression levels of the foreign gene in plants. Use of unusual plant codons in the foreign gene may result in stalling or stopping translation. Redesigning the coding sequence of the gene and/or removal of mRNA-destabilizing sequences can result in as much as 100-fold increases in expression levels (Strizov *et al.*, 1996). Optimization for plant codon usage of the gene expressing the heat-labile enderotoxin (LT-B) resulted in a 14-fold increase inexpression, but these levels stunted growth of the potato plants. The growth inhibition was overcome by targeting of the protein to tubers (Mason *et al*, 1998). Rouwendal *et al* (1997) enhanced expression of the *gfp* gene from *Aequorea victoria* in transgenic tobacco by resynthesising the gene to adapt its codon usage for that of plants (Rouwendal *et al.*, 1997). It has also been demonstrated that chloroplast genes transferred to the nuclear genome have adjusted to nuclear base composition and codon usage (Oliver, 1990)

1.4.3.5 Leader sequences

Mixed results have been obtained from use of specific leader peptides to target proteins to specific cell compartments or organs such as the endoplasmic reticulum, chloroplast or organs such as tubers. Several studies have found that targeting of proteins to the vacuole (Richter *et al*, 2000), and tubers resulted in increases in expression overcame the toxic effects of proteins already well expressed (Mason *et al.*, 1998). It is likely that increases in expression result from reduced degradation due to reduced proteolytic activity in seeds, chloroplasts, the endoplasmic reticulum and other targeted compartments. Some

studies have shown no increase (O'Brien *et al.*, 2000) or reductions in expression (Mason *et al.*, 1998) resulting from the use of targeting signals.

1.4.4 Foreign antigens produced in plants

Production of vaccines and antigens in plants is a rapidly expanding field of research. A huge variety of different antigens have been expressed in plants using the various systems described above. A summary of the foreign antigens produced in plants is given in table 1.3.

TABLE 1.3: Summary of foreign antigens expressed in plants

Potential application	Plant	Protein	Expression system	Reference
Hepatitis B virus	Tobacco	Recombinant Hepatitis B surface antigen	AMT	Mason *et al.* 1992, Thanavala *et al.* 1995; Tsuda *et al.*, 1998; Richter *et al.*, 2000;
	Lettuce			Kapusta *et al.*, 1999
	Potato			Kong *et al.*, 2001
Hepatitis C virus	Tobacco	HVR1 mimotopes/CTB	TMV	Nemchinov *et al.*, 2000
Murine hepatitis virus	Tobacco	Murine hepatitis epitope	TMV	Koo *et al.* 1999
Dental caries	Tobacco	Streptococcus mutant surface protein SpaA	AMT	Tacket *et al.*, 1999;
Autoimmune diabetes	Potato	*Vibrio cholera* toxin B subunit-human insulin fusion	AMT	Arakawa *et al.*, 1998 Lam *et al.*, 2000
		Glutamic acid decarboxylase	AMT	Ma *et al.*, 1997
Transmissible gastroenteritis coronavirus	Maize	Spike protein of TGEV Antigenic N-terminus of glycoprotein S (N-gS)	AMT	Streatfield *et al.*, 2001
	Potato			Gomez *et al.* 2000
Cholera and *E.Coli* diarrhoea	Tobacco/potato	*E.Coli* heat-labile enterotoxin LT-B	AMT	Lauterslager *et al.* 2001; Richter *et al.*, 2000; Tacket *et al.*, 1998
	Maize			Streatfield *et al.*, 2001
Oral vaccine against cholera	Potato	*V. cholera* toxin CtoxA and CtoxB subunits	AMT	Fischer *et al.*, 2000
Mucosal vaccines not requiring adjutants	Cow pea	D2 pepride of fibronectin-binding protein B of Staphylococcus aureus	CPMV	Brennan *et al.*, 1999
Norwalk virus	Tobacco/potato	Coat protein	AMT	Dixon *et al.*, 1997; Tacket *et al.*, 2000
Rabies	Tobacco/spinach	Rabies virus glycoprotein	AMT	McGarvey *et al.*, 1995
	Tobacco	Rabbies virus B-cell epitope	AIMV/TMV	Yusibov *et al.* 1997
HIV	Tobacco/ blackeyed bean	HIV epitope (gp120)	CPMV/AMT	Doran *et al.*, 2000; Mushegian *et al.*, 1995
	Cow pea	HIV epitope (gp 41)	CPMV	Brennan *et al.*, 1999
	Tobacco		*PVX	Marusic *et al.*, 2001
	Tobacco		AIMV/TMV	Yusibov *et al.* 1997
	Tobacco	p24	ATM	Zhang *et al.* 2002
	Tobacco		TBSV	Zhang *et al.*, 2000

Rhinovirus	Blackeyed bean	Human rhinovirus epitope (HR Doran *et al.*, 2000;)	CPMV	Tacket *et al.*, 1999
Foot and mouth	Blackeyed bean	Foot and mouth virus epitope (VP1)	CPMV	Tacket *et al.*, 1999; Beachy *et al.*, 1999
	Alfalfa		ATM	Dus Santos *et al.*, 2002 Wigdorovitz *et al.*, 1999 Carrillo *et al.*, 1998
	Arabidopsis		ATM	Wigdorovitz *et al.*, 1999
	Tobacco		TMV	
Canine parvovirus	Blackeye beans	VP2 peptide	*CPMV	Langeveld *et al.*, 2001 Nicholas *et al.*, 2002
	Arabidopsis	VP2 peptide (2121)	AMT	Gill *et al.*, 2001
Mink enteritis virus	Blackeyed bean	VP2 epitope	*CPMV	Dalsgaard *et al.*, 1997
Malaria	Tobacco	Malaria B-cell epitope	*TMV	Tacket *et al.*, 1999; Turpen *et al.*, 1995
Influenza	Tobacco	Hemagglutinin	TMV	Beachy *et al.*, 1999
Cancer	Tobacco	c-Myc	TMV	Beachy *et al.*, 1999
Bovine rotavirus A	Potato	VP6	AMT	Matsumura *et al.*, 2002
Human rotavirus	Tobacco		PVX	O'Brien *et al.*, 2000
Rabbit hemorrhagic disease virus	Tobacco	VP60	PPV	Fernandez-Fernandez *et al.*, 2001
Psedomonas Aeruginosa infections	Cow pea	Outer membrane F protein peptide	*CPMV	Brennan *et al.*, 1999a; Brennan *et al.*, 1999b
	Tobacco		*TMV	Gilleland *et al.*, 2000
			*TMV	Staczek *et al.*, 2000
Staphylococcus aureus	Cow pea	D2 peptide of fibronectin-binding protein B (FnBP)	*CPMV	Brennan *et al.*, 1999

*Chimaeric plant virus capsids

PPV: *Plum pox virus*; **TMV**: *Tobacco mosaic virus*; **CPMV**: *Cowpea mosaic virus*; **PVX**: *Potato virus X*; **AMT**: *Agrobacterium* mediated transformation; **TBSV**: Tomato bushy stunt virus; **AIMV**: *Alfalfa mosaic virus*; **CTB**: cholera toxin subunit B

1.4.5 Immunogenicity of plant derived antigens

The initial attempts to determine the immunogenicity of plant-derived antigens was disappointing and could be attributed to the impurities and low concentration of the plant-produced antigen. The hepatitis B surface antigen (HBsAg) expressed in transgenic tobacco formed VLPs and a crude extract containing 3% by weight of the HBsAg was used to immunize mice. However, the immune response was observed to be lower than that induced by the yeast-derived protein (Mason *et al.*, 1992). The expression was slightly higher (5 ng/g fresh weight) in lettuce and induced low levels of serum antibodies in humans (Kapusta *et al.*, 1999). Transgenic potatoes achieved a higher expression of HBsAg and oral administration of raw potatoes induced an antibody response that was greater than that required for protection (Kong *et al.*, 2001). About 25-50% assembly was

achieved for Norwalk virus capsid protein expressed in transgenic potato and immunization of volunteers with 150 g doses (215-751μg of VLPs depending on the batch) resulted in modest serum IgG increase after 3 weekly oral immunizations (Tacket *et al.*, 2000). Good protection against *E. coli* LT-B and transmissible gastroenteritis virus (TGEV) has been achieved using transgenic corn produced LT-B (5 or 50 μg) in mice and spike protein of TGEV (2mg) in piglets (Streatfield *et al.*, 2001). Immunization with a crude extract of transgenic *Arabidopsis*-expressed FMDV VP1 protein conferred complete protection (Carrillo *et al.*, 1998) whereas that expressed in alfalfa protected 12 of the 17 mice immunized (Wigdorovitz *et al.*, 1999). Human trial on plant-produced LT-B antigen of *E. coli* using 50-100μg of raw transgenic potatoes showed that antibodies to LT-B were detected in 10 of 11 volunteers fed with the plant-produced antigen and the antibody levels were similar to those obtained when volunteers were subjected to 10^6 infectious *E. coli* (Tacket *et al.*, 1998).

As an alternative to transgenic approaches, several pathogen-specific responses have been generated from purified transiently expressed recombinant virus. Chimaeric plant-produced vaccines displaying a parvovirus epitope (17 residue peptide from the S protein) on the coat protein of *cowpea mosaic virus* (CPMV) expressed in black-eyed beans (yield of 1-1.2mg of virus particles per g fresh plant material) were able to protect 11 of 12 animal immunized and subsequently challenged with MEV (Dalsgaard *et al.*, 1997). Transiently expressed FMDV VP1 in tobacco plants using TMV protected 30 mice immunized and challenged (Wigdorovitz *et al.*, 1999). It has also been demonstrated that virus-like particles of the flexuous plant virus PVX displaying the 2F5 ELDKWA epitope could induce high levels of HIV-1 specific IgG and IgA in mice immunized with recombinant VLPs (Marusic *et al.*, 2001).

These reports show the potential of plant-expressed vaccines as a cheap alternative to vaccine production systems. Evidence has not shown than any particular plant-based expression of foreign antigen method is best and success may depend more on the choice of antigen than on the method of expression and delivery (Awram *et al.*, 2002).

1.5 HIV NEF

1.5.1 Nef structure

All primate lentiviruses encode a *nef*; however, a related counterpart is absent from the genomes of the other animal lentiviruses (Luciw *et al.*, 1996). The *nef* gene of HIV-1 extends from the 3' end of *env* into the U3 domain of the 3' LTR. It differs from the HIV-2 counterpart in which the 5' end of *nef* overlaps the 5' end of *env* in a different translation frame.

The HIV-1 *nef* gene encodes 210 amino acids. Nef is translated from two multiply spliced early transcripts that are independent of the posttranscriptional function of Rev (Rev acts post-transcriptionally to regulate viral mRNA transport from the nucleus and splicing of long HIV transcripts which code for *gag, pol,* and *env* precursors). One transcript is monocistronic and the other is bicistronic and encodes both Rev and Nef (Schultz *et al.*, 1990). The 5' ends of most HIV-1 *nef* genes have two initiation codons corresponding to Met1 and Met20. Both initiation codons are used and the two forms of Nef are detectable in infected cells (Kaminchik *et al.*, 1994). Analysis of Nef proteins in cells infected with diverse virus isolates has been done by electrophoresis in polyacrylamide gels under denaturing conditions (Ugner *et al.*, 1992). Such analyses revealed a huge amount of intra- and inter-patient heterogeneity in the size of the Nef proteins ranging from 27 to 34 kDa. This heterogeneity is attributable to variations in sequence as well as differences in post-translational modifications.

1.5.1.1 Secondary structure

Comparison of the predicted amino acid sequences of HIV and SIV Nefs reveals both conserved and variable regions (Sherman *et al.*, 1990). Figure 1.2 shows the most highly conserved features of the Nef proteins of HIV-1, HIV-2 and SIV. The most prevalent form of the protein is that which is initiated at the codon corresponding to a methionine at position 1. The second amino acid is a conserved glycine residue. In infected cells, Met1 is removed and and myrictic acid is covalently linked to G2 at the new N-terminus. This

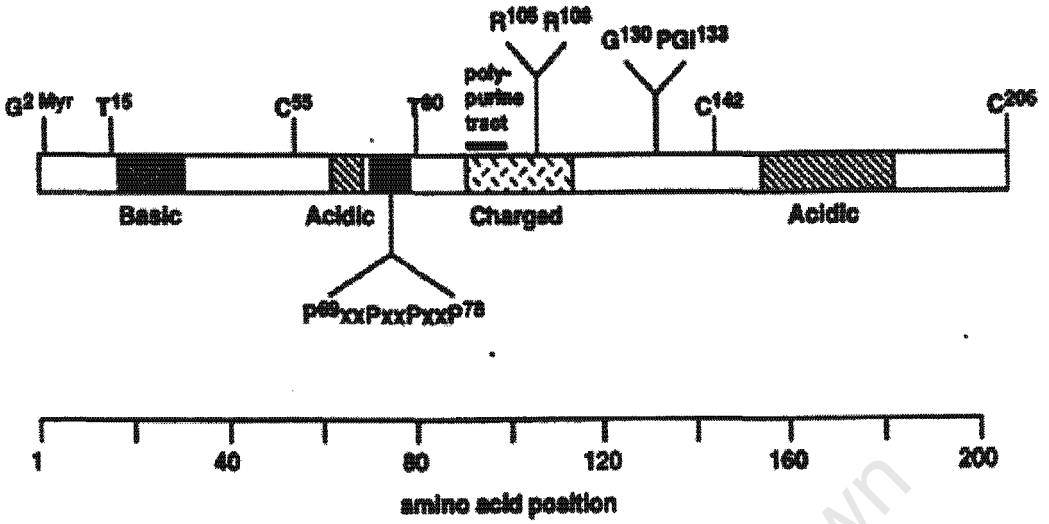


Figure 1.2. Predicted structural features of Nef. The diagram shows conserved features of the Nef secondary structure. The diagram is taken from Luciw *et al.*, 1996.

myristoylation was shown to be essential in determining the sub-cellular distribution of Nef and has important implications on the normal function of the protein (Gang *et al.*, 1992). Immunochemical and biochemical analysis of infected cells and cells transfected with expression plasmids show that Nef is predominantly localised to the cytoplasm and the inner surface of the cell plasma membrane (Gang *et al.*, 1992). Mutation of the G2 residue prevents myristoylation and disrupts subcellular targeting of the protein to the cell membrane. Such mutant proteins are defective in the observed serine kinase activity associated with functional Nef (Sawai *et al.*, 1995) and are unable to carry out other Nef functions (Gang *et al.*, 1992). HIV-1 Nef also has a threonine residue in position 15. This residue has been shown to be a target for phosphorylation by protein kinase C (Guy *et al.*, 1990). This residue however, is not highly conserved. Other threonine and serine residues in Nef were also shown to be phosphorylated by this kinase, a case in point being Thr80 (Kaneko *et al.*, 2000). The sequence of amino acids that follows this is usually rich in basic amino acids for the next 15 or so residues (Luciw *et al.*, 1996). Sequence comparison of independent HIV-1 isolates reveals a two to 14 amino acid insertion in this region at residue 24. These insertions are generally characterised by charged amino acids and include one or more proline residues (Luciw *et al.*, 1996). A highly conserved acidic region is located at position 62 to 65 of HIV-1 Nef and this region is predicted to be on

the surface of the folded protein because of its hydrophilicity. The region between positions 69 and 80 of HIV-1 Nef contains repeats of the sequence Pro-Xa-Xa-Pro (Luciw *et al.*, 1996). This proline motif is a feature of proteins that interact with the *src*-homology region-3 (SH3) domain of several members of several tyrosine kinases involved in signal transduction (Saksela *et al.*, 1995). The central region of Nef is conserved in primate lentiviruses (Luciw *et al.*, 1996). This region consists of a stretch of about 30 amino acids, the majority of which have charged side groups and are predicted to lie on the surface of the folded protein. This conserved central region contains a sequence of six codons that overlaps a 16-nucleotide stretch of A and G residues. This polypurine tract is highly conserved and functions as a primer in reverse transcription (Luciw *et al.*, 1996). Many HIV-1 Nef isolates contain the sequence G130-P131-G132-I133/V133. This sequence is predicted to form a β-turn. Nef proteins of HIV and SIV have a leucine repeat in the middle and an acidic domain near the C-terminus. These features are reminiscent of leucine zippers and activation domains of the acidic class of transcription factors, which bind DNA (Luciw *et al.*, 1996).

1.5.1.2 Tertiary structure

HIV-1 Nef has three conserved cysteine residues (C55, C142 and C205) which are reported to form intra-chain disulphide bonds (Zazopoulos *et al.*, 1993). Studies have shown that the N-terminal residues of Nef are generally unstructured (Grzesiek *et al.*, 1997). This region of the protein is generally rich in glycine residues and the structure is therefore not influenced by side groups, which would limit rotation around the peptide bond (Luciw *et al.*, 1996). This region of the protein is cleaved by a viral protease between amino acid residues W57 and L58 generating two fragments of ~7kDa and ~20kDa (Grzesiek *et al.*, 1997). In addition to their role as a target site for the HIV-1 protease, residues that compose the cleavage site (W57 and L58) have been shown to participate in an intra-molecular interaction with a hydrophobic pocket formed by two alpha helices in the well-folded Nef core (Pandori *et al.*, 1998). Cleavage therefore releases the well-folded Nef core from the amino terminal membrane anchor potentially freeing the Nef core for action within the virion or target cell. This core contains many of

the regions that are crucial for many of the Nef functions and has been the subject of several studies involving X-ray crystallography and nuclear magnetic resonance (NMR) (Grzesiek *et al.*, 1997).

The core region of HIV-1 Nef contains two large helices, H1 and H2 spanning residues Y81 to K94 and R105 to H116 respectively (Grzesiek *et al.*, 1997). There are two smaller helical regions H3 and H4 of 4 and 5 amino acids in length starting at S187 and M194. In the unmodified Nef protein the C-terminal end of the flexible region between residues 60 and 68 is attached to the rest of the protein at the hydrophobic pocket between helices H1 and H2. HIV-1 Nef contains an extended, five stranded anti parallel β sheet consisting consecutively of B1, B5, B4, B3 and B2. Strand B1 consists of residues L100, I101 and H102. B2 consists of residues N126, Y127, T128 and P129. B3 is made up of residues I133, R134, Y135, P136 and L137. B4 comprises residues Y143, K144, L145, V146 and P147. B5 is the longest of the β-strands and consists of residues L181, E182, W183, R184, F185 and D186 (Grzesiek *et al.*, 1997).

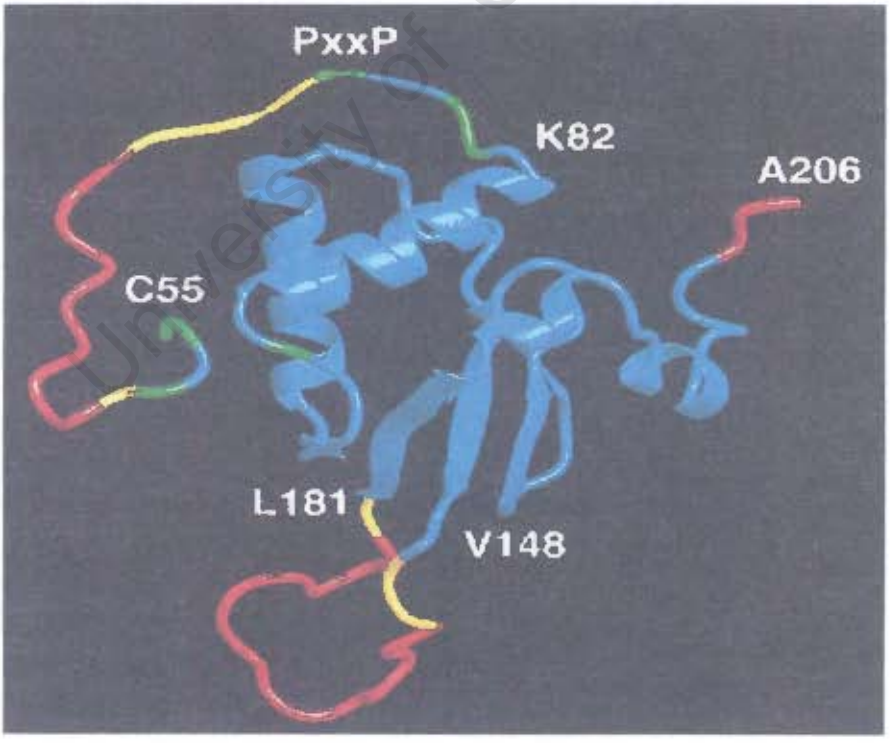


Figure 1.3. Backbone representation of the refined structure of the Nef core. This graphic was generated with the program MOLMOL and is taken from Grzesiek *et al.* (1997).

The arrangement of strands and helices in the Nef protein appear to be somewhat irregular in nature. In particular, the large separation between helices H1 and H2 forms a hydrophobic solvent exposed crevice. This structure is important in the binding of Nef to the SH3 domain of several cellular proteins and for interaction with CD4 (Grzesiek *et al.*, 1997). This open appearance of the protein led to the suggestion that the protein core would not be particularly stable. However, further detailed studies of the solution structure and backbone dynamics using ^{15}N relaxation data do not support this suggestion (Grzesiek *et al.*, 1997). Residues that form parts of HIV-1 Nef helices and strands have heteronuclear nuclear Overhauser effects NOEs of ~0.74, which is close to values observed for well ordered regions in "stable" proteins such as staphylococcal nuclease (Grzesiek *et al.*, 1997). A backbone representation of the refined structure of Nef is shown in figure 1.3.

Experiments with HIV-1 Nef have revealed that the protein has a dimerisation constant that remains close to the concentration of the protein used. This means that ~50% of the molecules are in the monomeric form, and 50% are dimeric. Nef has also shown a tendency to form aggregates at millimolar concentrations, especially at pH values below 8 (Grzesiek *et al.*, 1997).

1.5.2 NEF Functions and its importance as a molecular target for drug and vaccine development

The function of Nef is somewhat obscure. Indeed over the years several studies have presented contradictory results. Some initial studies appeared to demonstrate that this gene was of little importance in the life cycle of the virus while other studies assigned critical roles in the virus life cycle to this gene. In some early studies it was suggested that Nef acts as a negative factor in virus replication by repressing the transcription from the LTR (Ahmad *et al.*, 1988) hence it received its acronym "negative factor". Effective analysis of Nef function has been made more difficult by the general observation that this viral gene product is dispensable for efficient replication of both HIV-1 and SIV in culture (Hua *et al.*, 1997). Furthermore, virus from some HIV-1 infected long term non-progressors have been shown to have deletions within the *nef* gene (Micheal *et al.*, 1995).

Thymic atropy and loss of T-cells in peripheral lymphoid organs with resultant immunodeficiency, has been seen in transgenic mice expressing the nef gene alone (Hanna *et al.*, 1998) suggesting that Nef could be directly responsible for the depletion of lymphocytes and thymocytes during HIV infection.

Further studies revealed that although variations in experimental conditions may give conflicting results. Nef is able to induce virus replication through T-cell activation in primary quiescent CD4 cells (De Ronde *et al.*, 1992), is needed for maintaining high virus loads in persistent virus infections (Kestler *et al.*, 1991), is the major disease determinant in transgenic mice (Hanna *et al.*, 1998) and may contribute to the neuropathogenesis seen in AIDS patients (Koedel *et al.*, 1999). These effects are enabled by at least four activities associated with Nef: induction of CD4 and MHC class 1 down-regulation, enhancement of viral infectivity and alterations in cellular signaling pathways (Azad *et al.*, 2000)

1.5.2.1 Cytopathic effects of Nef

A number of studies suggest that the cytopthic effects of Nef could be related to its being targeted to the plasma membrane and other membranes by the sequence at the N-terminus, which has an N-myristoyl group attached at Gly-2. Full length recombinant Nef and N-terminal peptides of Nef but not truncated Nef starting at the second methionine at position 19, possess membrane perturbing properties and are capable of fusing phospholipid vesicles (Curtain *et al.*, 1994).

The N-terminal region of Nef shows striking homology and similarity in tertiary structure to bee venom mellitin and when myristylated at the N terminus has a dramatic disordering effect on lipid bylayers (Curtain *et al.*, 1994). Full length myristylated Nef is released into the extracellular medium when expressed in yeast (Macreadie *et al.*, 1995) or mammalian cells (Guy *et al.*, 1990), and is expressed on the cell surface of baculovirus-infected insect cells (Fujii *et al.*, 1996). The yeast derived extracellular Nef is present as a complex with membranous material (Macreadie *et al.*, 1995), and the addition of the culture medium containing Nef to naïve yeast cells leads to their death as

measured by their inability to form colonies on culture plates. These activities of the myristylated N-terminal region of Nef and its structural similarity to mellitin suggested the interesting possibility that when present in the extracellular medium it could be lytic to human cells.

Addition of myristylated N-terminal peptides of Nef to the extracellular medium of human erythrocytes, CD4+ CEM cells or PBMC, caused very rapid haemolysis or cell lysis in a dose dependent manner (Curtain *et al.*, 1997). This effect has been seen with erythrocytes, monocytic cell lines, a variety of yeast and bacterial cells (Lowe *et al.*, 1997). It is evident that the mysitylated N-terminal Nef peptide-mediated cell lysis is quite promiscuous suggesting that extracellular Nef-induced ctyotoxicity for eukaryotic and prokaryotic cells is exerted through a common effect on cell membranes (Azad *et al.*, 2000). The myristylated N-terminal Nef peptide-mediated death of a variety of cells could, at least in part explain the deaths of various cell types within lymphoid organs during HIV infection (Lowe *et al.*, 1997). A role for soluble or cell surface associated Nef in bystander cell killing has also been suggested by others

1.5.2.2 Modulation of cell surface receptors

a) CD4 Downregulation

In CD4+ cells, Nef is able to down-regulate the expression of CD4 mainly by enhancing its endocytosis as well as lysosomal degradation, which leads to a decrease in the half life of this protein, however, synthesis and intracellular transport of CD4 molecules are not affected by Nef (Aiken *et al.*, 1994, Anderson *et al*, 1994) A dileucine-based sorting signal (aa 160-165 E/DXXXLL) in Nef is used to address cellular sorting machinery (Craig *et al.*, 1998). Nef functions in a multistep process, first by dissociating the CD4-$p56^{lck}$ complex that leads to the exposition of an endocytosis motif present in the intracellular domain of CD4 (Bandres *et al.*, 1995). Thereafter Nef connects CD4 to clathrin-containing adapter complexes which function as vesicle coat components in different membrane traffic pathways (Greenberg *et al.*, 1997). Finally Nef targets internalized CD4 molecules to degradation by connecting CD4 to β-COP protein present in endosomes (Piguet *et al.*, 1999). Various other cellular proteins including vacuolar

ATPase, phopatidylinositol-3-kinase and p35 thioesterase may be involved in Nef induced CD4 andocytosis (Liu *et al.,* 1997). In addition to the process described above, Nef is also able to suppress the function of a novel protein Naf1, which increases the cell surface CD4 expression (Fukushi *et al.,* 1999). At least when strongly overexpressed in transient transfection systems, extracellular CD4 molecules have been shown to be deleterious for budding virions, either by inhibiting HIV-1 progeny virion release by binding to env proteins (Ross *et al.,* 1999) or by decreasing the amount of Env incorporated, thus making the virion less infective (Lama *et al.,* 1999). Hence the purpose of Nef induced CD4 downregulation appears to enhance the budding or infectivity of virus particles.

b) MHC class 1 Downregulation

The Nef induced MHC class 1 and CD4 endocytoses are separate processes: the domains involved in the MHC class 1 downregulation consists of an N terminal alpha-helix (aa 17-26), an acidic stretch (aa 62-66) and a Pro-rich (aa 69-78) segment of Nef, whereas CD4 binding and downregulation is mediated through several amino acids in the core region of Nef (most importantly: aa WLE 57-59, GGL 95-97, RR 105-106, L 110, D 123, EE 154-155, DD 174-175) (Aiken *et al.,* 1996, Liu *et al.,* 2000). In addition, Nef targets the MHC 1 protein to the trans-Golgi network by connecting the cytoplasmic tail of MHC 1 to the PACS-1 dependent protein sorting pathway (Piguet *et al.,* 2000). Decreasing the amount of MHC class 1 molecules on the surface is one mechanism that HIV-1 uses to escape CTL response directed against virus infected cells (Collins *et al.,* 1998)

1.5.2.3 Enhancement of viral replication and infectivity through alterations in cellular signaling pathways and modulation of apoptosis

Within a few hours of infection of cells, Nef stimulates the reverse transcription on proviral DNA (Aiken *et al.,* 1995). Nef enhances the infectivity of virions in a producer cell-dependent manner Tokunuga *et al.,* 1998) suggesting that interactions with cellular factors are needed for this process. The Pro-rich sequence (aa 69-78), a conserved RR

motif (aa105-106) and the dileucine motif (aa 160-165) are needed for optimal viral infectivity (Craig *et al.,* 1998).

Nef has several effects on cellular signaling pathways. First, through its well conserved Pro rich motif it is capable of binding to the SH3 domains of several Scr family tyrosine kinases including Hck, Lyn, Lck, Fyn (Lee *et al.,* 1996). The Hck kinase activation caused by Nef has been reported to cause malignant transformation of fibroblasts (Briggs *et al.,* 1997). In CD4+ cells, Nef's association with Lck causes suppression in the essential function of Lck in the activation induced apoptosis. This delays cell death during HIV infection, thus allowing increased virus production. Nef also delays apoptosis by inhibiting a p53 dependent apoptosis pathway through its interactions with that protein (Azad *et al.*, 2000). That interaction is dependent on the N terminal regions of both proteins.

Secondly, Nef associates with serine kinases, including various isoforms of protein kinase C (Smith *et al.,* 1996), members of the mitogen activated-protein kinase (MAPK) pathways (Greenway *et al.*, 1995) and PAK2 (Renkema *et al.*,1999). Nef may also effect on PAKs indirectly by activating their regulators (Liu *et al.*, 2000).

Thirdly, Nef may alter Ca2+ homeostasis in a variety of cells (Manninen *et al.,* 2000). Finally, the direct binding of Nef to an important component of T-cell receptors may lead to activation of healthy T-cells without antigen stimulation, apoptosis due to the Fas-ligand induction and T-cell receptor down-regulation thereby further protecting infected cells (Xu *et al.*, 1999)

1.5.2.4 Destruction of the humoral immune response

Nef causes B-cell dysfunction by several mechanisms. Firstly, the gradual destruction of CD4+ helper T-cells will lead to impairment of B-cell function as well. Secondly, biased or restricted antibody V-region gene usage may limit the available repertoire of antibodies against HIV-1 and opportunistic infections (Muller *et al* 1993). Thirdly, the elevated levels of B-cell bearing IL-6 receptor in HIV-infected subjects, together with enhanced production of IL-6 (Boue *et al.,* 1992), can contribute to the

hypergammaglobulinemia. Finally, the decreased level of CD70, a TNF-related transmembrane protein induced by the activation of lymphocytes on B-cells fron HIV infected subjects is involved in the low IgG production after T-cell antigen stimulaton (Wolthers *et al.*, 1997). Changes in expression of other cytokines and cytokine receptors may also contribute to the destruction of the humoral response (Wolthers *et al.*, 1997).

1.5.3 Nef as a vaccine candidate

1.5.3.1 Therapeutic and Prophylactic Implications

Therapeutic strategies that prevent the death of bystander cells while promoting the early death of HIV infected cells could play an important role in the preservation of the immune system, and would be a useful addition to currently used antiviral strategies. This could be achieved by targeting Nef. The evidence given above suggests that Nef can cause death when present in the extracellular medium, but when inside infected cells it seems to protect the cell from apoptotic death. Rationally designed inhibitors that bind specifically to the membrane active regions of Nef could stop extracellular Nef from binding to and permeabilizing cell membranes and thereby prevent Nef induced cell death. Specific antibodies that bind to these regions of Nef could also be important therapeutic or prophylactic agents (Azad *et al.*, 2000). Because Nef can also inhibit apoptosis and act as a positive factor for for increased virus production, therapeutic approaches that reverse the Nef-induced inhibition of apoptosis of infected cells could result in selective elimination of infected cells (Azad *et al.*, 2000).

1.5.3.2 Epitopic Regions of Nef

The Nef protein is very immunogenic and is rich in CTL epitopes. Several studies have focussed on characterising these regions. Identification of the epitopic regions in HIV-1 Nef has been carried out by administering HIV-1 *nef* DNA sequences epidermally in mice transgenic for the human major histocompatability complex (MHC) class 1 molecule, HLA-A201 (Sandberg *et al.*, 2000). Ten potential HLA-A2 binding 9-mer Nef peptides were identified by a computer-based search algorithm. By a cell surface MHC class 1 stabilisation assay, four peptides were scored as good binders, whereas two peptides bound weakly to HLA-A2. After DNA immunization, cytotoxic T lymphocyte

(CTL) responses were predominantly directed against the *NEF* 44-52, 81-89 and 85-93 peptides. The 45-52 and 85-93 peptides as well as the 139-147 peptides also generated specific CTL responses to peptide immunization (Sandberg *et al.*, 2000).

A more recent study by Mashishi *et al.*, (2001) sought to identify the epitope specific CD8+ T cell responses to subtype C Nef as these would be important in the development of a subtype specific vaccine. Anti-Nef CD8+ T cell responses were identified in recent HIV-1 subtype C infected individuals from Southern Africa. A combination of the IFNγ ELISPOT assay and intracytoplasmic cytokine staining were used to identify regions within Nef containing CTL epitopes. Samples were obtained from 69 individuals within their first year of infection and screened with a subtype C-based set of overlapping peptides synthesised based on a nef sequence from prevailing virus in South Africa.

The results of this study showed that 70% of the individuals recognized Nef, with 75% of responders targeting three epitope regions. Twelve individuals (25%) recognised the peptide PGPBVRYPLTFGWCF with a mean frequency of 958 ±543 sfu/10e6 PBMC; eight individuals (15%) recognised the peptide PVRPQVPLRPMTYKA with a mean frequency of 762 ±576 sfu/10e6 PBMC and fifteen individuals (31%) recognised the peptide YKAAFDLSHFLKEKG with a mean frequency of 802 ±586 sfu/10e6 PBMC. HLA-restriction assays show this epitope to be restricted by both HLA-A*02 and –A*30, akin to the RSLYNTVATLY epitope in p17 Gag. Out of the three epitope responses, there was a hierarchy of immunodominance, where exclusive recognition of the YKAAFDLSHFLKEKG was seen in 1/3 of responders; PGPBVRYPLTFGWCF by 1/5 and PVRPQVPLRPMTYKA by 1/10 responders.

These data show that early infected individuals with subtype C HIV-1 display a highly focussed CD8+ Tcell response to Nef. Three immunodominant epitope regions consisting 75% nef responses were found within a narrow span of 73 amino acids. This immunogenic region is highly conserved between subtypes A, B and D, making it desirable to include in vaccine candidates (Mashishi *et al.*, 2001).

1.6 SELECTION OF VIRAL STRAINS SUITABLE FOR INCLUSION IN HIV-1 CANDIDATE VACCINES FOR SOUTHERN AFRICA

Viral strains used in the design of a vaccine for southern Africa need to be shown by genotypic analysis to be representative of the circulating strains, and not of unusual outlier strains. In addition, it is important that a vaccine strain has the phenotype of a recently transmitted virus, which is non-syncytium inducing (NSI), macrophage tropic and uses the CCR5 co-receptor for entry (R5 viruses). R5 viruses are the most commonly transmitted viruses and predominate in patients with advanced AIDS. In order to select this phenotype, many vaccine developers have focussed on using viral strains from cohorts with high incidence rates where dates of seroconversions are known (Williamson *et al.*, 2003). In South Africa, a well established sex-worker cohort in KwaZulu-Natal was used to select appropriate strains for development of an HIV vaccine. Viral isolates from 15 acutely infected individuals were sequenced in the *env, gag* and *pol* genes and were phenotypically characterised. These sequences were compared with a similar collection from asymptomatic individuals from the Gauteng region as well as other published subtype C sequences in the Genbank database, including sequences from other southern African countries. Two potential vaccine strains – DU151 and DU422 – were selected from the acute infection cohort: the selection was based on amino acid homology to the consensus in all three regions, CCR5 tropism and ability to replicate in tissue culture. The complete genomes of these isolates have also been sequenced and shown to be HIV-1 subtype C throughout, with no evidence of recombination (Williamson *et al.*, 2003). The strain selected for use in this thesis is the DU151 strain.

1.7 OBJECTIVES OF THE PROJECT

Nef has been expressed in many systems but no literature could be found showing the expression of Nef in plants. The aim of this project was to investigate the production of HIV-1 subtype C Nef in plants using a TMV-based plant virus vector pBSG1057 in *Nicotiana benthamiana*. The production of this protein in plants could provide a potential cheap source of antigen that could act as part of a candidate vaccine. Primarily,

expression of Nef as a free protein was to be investigated. The significance of codon usage on expression levels was to be studied by comparative analysis of expression of a wild type gene against a synthesized *nef* gene reflecting plant codon bias. Expression of free Nef in *Nicotiana benthamiana* would be preceded by rapid expression of Nef protein in an *E. coli* expression system. This bacterially expressed protein would be used to develop immunoassays for detection and quantification of Nef in *Nicotiana benthamiana*. The second part of the project would investigate expression recombinant TMV particles decorated with immunodominant peptides from the Nef core region harbouring CTL epitopes, with the aim of purifying them and possibly investigating their immunogenicity. Two different peptides would be selected from the Nef core region based on their ability to stimulate CTL responses. Oligonucleotides encoding the each of the peptides would be fused to the N or C-termini of the TMV *cp* gene to be expressed as translational fusions. Expression of the decorated capsid proteins and their ability to assemble into rTMV rods would be investigated. In the event that such particles were produced a further aim was to purify characterize them physically and possibly study their immunogenicity in a small animal model.

CHAPTER 2

Expression of HIV-1 Nef in *E. coli*

2.1 INTRODUCTION

HIV-1 Nef has been successfully over-expressed in several systems including yeast (Macreadie *et al.*, 1995) mammalian cells (Guy *et al.*, 1990) and baculovirus infected insect cells (Fujii *et al.*, 1996). As outlined in the previous chapter, the use of *E. coli* as an expression system benefits from the bacteria's well characterized genetics and the availability of a large number of cloning vectors (Baneyx *et al.*, 1999). The bacterium can also be grown rapidly at high density on inexpensive substrates. This advantage outweighs the concern that overproduction of heterologous protein in the cytoplasm of *E. coli* is often accompanied by misfolding and segregation into insoluble aggregates known as inclusion bodies. This means that there is no guarantee that the *in vitro* refolding will yield large amounts of biologically active products. However, the production of Nef in its native, active condition was not a priority for this project although such a protein might improve the accuracy of the assay that would be developed using bacterially expressed Nef as a control reagent.

Several studies have reported procedures to purify recombinant Nef. Glutathione-S-transferase (GST)-tagged Nef was purified to homogeneity in its native conformation using glutathione sepharose matrix. However, given the size of the GST tag and its potential effect on the overall protein structure, this approach requires a proteolytic cleavage step which can affect protein recovery (Lee *et al.*, 1996). In contrast Nef or His-tagged Nef (His(6)-Nef) was purified as such using heparin affinity column (Kohleisen *et al.*, 1996) or immobilized metal ion affinity chromatographies (IM-ACs) (Federico *et al.*, 2001) respectively. IMAC is a very powerful method for purifying recombinant proteins with histidine affinity tails in their native state or in denaturing conditions. In contrast to GST tag, the relatively small size of the His tails have in general, minimal effects on the overall structure and biological functions of proteins (Janknecht *et al.*, 1997).

The pProEX™ HT Prokaryotic Expression System (Life Technologies™) is designed for the expression of foreign proteins in E. coli. The protein is expressed fused to a 6 histidine sequence $(His)_6$ for affinity purification. The gene of interest is cloned into the multiple cloning site of either pProEX™ HTa, pProEX™ HTb, or pProEX™ HTc. The

DNAs differ from each other with respect to their reading frames relative to the 6x histidine affinity tag. The *trc* promoter and *lacI*^{q} gene enable inducible expression of a cloned gene with IPTG. Upon expression, the histidine sequence is at the amino terminus of the fusion protein. The histidine sequence has strong affinity for Ni-NTA resin matrix making it simple to purify the desired protein. The plasmids also contain the pBR322 origin of replication, the β-lactamase gene conferring ampicillin resistance.

Rapid expression and purification of HIV-1 Nef using this system would provide a reagent that could be used to develop immunological diagnostic assays for detecting Nef in plants. The reagent could also be used for generating anti-Nef antibodies should the need arise. That would however be beyond the scope of this project as several anti-Nef antisera are available commercially.

2.2 MATERIALS AND METHODS

2.2.1 Amplification and subcloning of nef gene.

The complete genome of the HIV-1 subtype C isolate DU151 from a recently seroconverted acutely infected individual was obtained as a cloned and sequenced DNA pDU151 from Dr Joanne Van Harmelen at The Institute of Infectious Diseases and Molecular Medicine (IIDMM) at the University of Cape Town's Faculty of Health Sciences. To alleviate safety concerns arising from the extremely cytotoxic nature of the N-terminal region of the Nef protein as reviewed in the literature survey, it was decided that all work on this gene should exclude the first 59 codons of the gene.

Two synthetic oligonucleotides to be used as PCR primers were constructed, based on the published sequence of DU151.

The upstream primer (Fprim) 5'*CCTTAATTAAGGATCC***ATG**CAAGCACAAGAGGAGGAAC 3' was constructed to include 19 bases of the *nef* sequence (underlined) which would anneal 177 bases into the *nef* sequence thereby eliminating the first 59 codons of the Nef ORF. A *Pac* I site for

cloning into pBSG1057 and two added cytosines on the 5' end to promote *Pac* 1 digestion once cloned into the intermediate vector were also included. A *Bam* HI site for cloning into pProEX HT and an ATG start codon (in bold) for transcription of the cloned *nef* sequence were also included.

The downstream primer Rprim (5' *CGGCTCGAG*TCAGCAGTCTTTGTAATACTCC 3') was constructed to anneal to the 3' end of the *nef* gene (underlined). An *Xho I* site for cloning into pBSG1057 and pProEX HT, one cytosine and two guanosine nucleotides to promote *Xho* 1 digestion were also added.

PCR amplification was carried out as outlined in appendix A3 and the resulting product was gel purified and ligated into the pGem-T Easy (Promega®) vector to give pGemNef. pGemNef was used to transform high efficiency DH5α competent cells (appendix A8) and plated onto LA plates with ampicillin. Resulting colonies were selected and grown overnight at 37 C in 5ml LB with ampicillin. Plasmid DNA was extracted by miniprep. Extracted DNA was analyzed for the presence of the required insert by restriction enzyme digestion with *Pac* I and *Xho* I from (Roche® Biochemicals) and their appropriate buffers. Restriction digests were analyzed by agarose gel electrophoresis.

Two clones which showed the expected clavage patterns with *Pac* 1/*Xho* 1 digests were subjected to large scale plasmid DNA extraction using the Nucleobond™ kit (Machery Nagel) according to the manufacturer's instructions. Purified plasmid DNA was sequenced by a modification of the dideoxy chain termination method of Sanger *et al.* (1977) for dsDNA templates using the universal –80 forward primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3'; Pharmacia Biotech) and –80 reverse primer (5'-GAGCGGATAACAATTTCACACAGG-3'; MWG Biotech) fluorescent-labeled Cy5-Far Red primers from the Thermosequence™ cycle sequencing kit (Amersham Life Science). Cycle sequencing reactions were performed on an ALF-Express™ DNA Sequencer version AMV2.0 (Pharmacia Biotech). Labeling and termination reactions were carried out in the presence of 7-Deaza dGTP. Analysis of generated sequences was done using DNAMAN for Windows, version 4.13 (Lynon Biosoft, ©1994-1996)

2.2.2 Cloning of *nef* into pProEX™ HT

0.5µg pProEX HTb DNA was digested with *BamHI* and *XhoI*. The digested DNA was electrophoresed in a 0.8 % TBE agarose gel and the band containing the digested DNA excised from the gel and the cut plasmid DNA was purified. 0.5µg pGemNef DNA was digested with *BamHI* and *XhoI*. The digested DNA was electrophoresed in an agarose gel and the band containing the excised *nef* DNA was excised from the gel and the desired DNA was extracted.

The *nef* DNA and the cut pProEX HTb DNA were ligated overnight at 4°C in a vector:insert volume ratio of 1:8 with 2 units T4 DNA ligase in ligation buffer (Promega). DNA from the ligation reaction was used to transform competent DH5α *E. coli* (appendix A8). Plasmid DNA from resulting colonies was extracted after growing overnight cultures by the miniprep method using reagents and materials from the Roche® Hydrobond™ plasmid DNA extraction kit. The nature of the DNA was determined by restriction endonuclease digestion with *BamH* I and *Xho* I and gel electophoresis (appendix A5). The plasmid containing the cloned *nef* gene was designated pProNef.

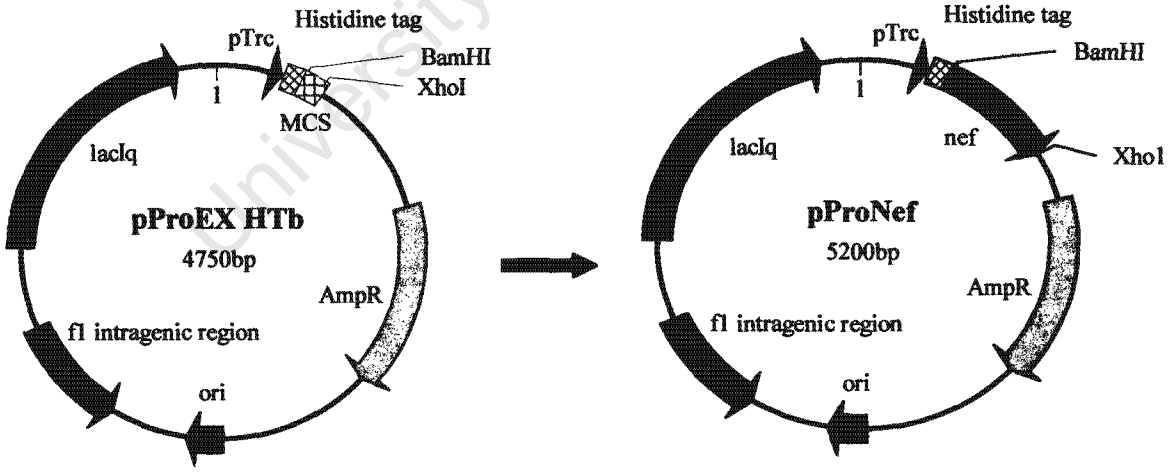


Figure 2.1: Plasmid maps showing the cloning procedure to create pProNef

2.2.3 Small scale induction of Nef protein expression in *E. coli*

A single colony of pProNef was used to inoculate 2ml of LB media with 100µg/ml ampicillin. This culture was incubated overnight at 37°C with agitation. The following day, 10ml of LB media with 100µg/ml ampicillin was inoculated with 0.1ml of the overnight culture and grown at 37°C with agitation. Once the culture reached an OD (A_{590}) of between 0.5 and 1.0, 1ml was removed and centrifuged for 1 minute in a microcentrifuge. The supernatant was discarded and the pelleted cells were resuspended in 100µl of PBS. This was the uninduced sample. To the remaining culture, IPTG was added to a final concentration of 0.6mM and the culture was returned to 37°C with agitation. 1ml aliquots of cells were removed at 1, 2 and 3 hours after induction and the A_{590} measured. The cells were centrifuged for 1 minute in a microcentrifuge and the pellets resuspended in 100µl of PBS. These were the induced samples. 0.2 A_{590} of each sample was placed in a separate microcentrifuge tube and mixed with an equal volume of 2X SDS sample buffer. The samples were then boiled for 10 minutes and resolved by SDS-polyacrylamide gel (5% stacking and 15% resolving) electrophoresis and the resulting gel was stained with 1% Coomassie brilliant blue (in 45% methanol and 10% acetic acid) and destained by repeated changes of 45% methanol and 10% acetic acid (appendix A12).

2.2.4 Large Scale Induction of Nef Protein in *E. coli* Expression

A single colony of recombinant pProNef was used to inoculate 10ml of LB media with 100µg/ml ampicillin. The inoculated media was incubated overnight at 37°C with agitation. 5ml of this overnight culture was used to inoculate 500ml of LB media with 100µg/ml ampicillin. The culture was incubated at 37°C with agitation until it reached an OD (A_{590}) of between 0.5 and 1.0. At this point a 1ml sample was removed as an uninduced control. The remainder of the culture was induced with IPTG to a final concentration of 0.6mM and incubated at 37°C with agitation for 3 hours. After 3 hours the cells were harvested by centrifugation at 10000 x g for 10 minutes. The supernatant fluid was decanted and the wet weight of the resultant pellet determined. The pelleted cells were then stored and -20°C prior to protein purification.

2.2.5 Purification of Inclusion Bodies

Pelleted bacterial cells were resuspended in 4 volumes of lysis buffer [20mM Tris-HCl (pH 8.5 at 4°C), 5mM 2-mercaptoethanol, 1mM PMSF]. The suspension was then treated with lysozyme to a final concentration of 100mg/ml and left for 30 minutes at room temperature. The lysozyme treated suspension was then divided into 1ml volumes in microcentrifuge tubes and sonicated until 80% cell lysis was achieved. The degree of lysis was measured by overall drop in A_{590} of the suspension. This crude extract was pooled and centrifuged at 15000 x g for 20 minutes.

Inclusion bodies were purified and washed by the method of Sambrook *et al.* (1989). The pellet fraction of the crude extract was resuspended in 9 volumes of lysis buffer [20mM Tris-HCl (pH 8.5 at 4°C), 5mM 2-mercaptoethanol, 1mM PMSF] containing 0.5% Triton X-100 and 10mM EDTA (pH 8.0) and incubated for 5 minutes at room temperature. The suspension was centrifuged at 12000g for 15 minutes in a microcentrifuge. The supernatant was decanted and the pellet resuspended in 100µl of water. The washed pellet was resuspended in 100µl of lysis buffer containing 8M urea and incubated for 1 hour at room temperature. This solution was added to 9 volumes of 50mM KH_2PO_4 (pH 10.7), 1mM EDTA (pH8.0), 50mM NaCl, and held at room temperature for 30 minutes with the pH adjusted to 10.7 with KOH. The pH was lowered to 8.0 with HCl and the suspension stored at room temperature for 30 minutes. The suspension was centrifuged at 12000g for 15 minutes at room temperature in a microcentrifuge. The supernatant was removed and both fractions analysed by SDS-PAGE to determine the degree of inclusion body solubilization.

2.2.6 Purification of Nef by Affinity Chromatography

This procedure was carried out with the intention of purifying the Nef protein while simultaneously refolding the protein by gradually reducing the urea concentration. The affinity chromatography column was loaded with 2ml of Ni-NTA resin (Life Technologies™) and equilibrated with buffer A [20mM Tris-HCl (pH 8.5 at 4°C),

100mM KCl, 5mM 2-mercaptoethanol, 10% glycerol, 20mM imidazole] containing 8M urea, while maintaining a flow rate of 0.5ml/minute. The solubilised protein was loaded onto the column. The column was washed successively with 2ml volumes of buffer A containing 8M, 7M, 6M, 5M, 4M, 3M, 2M, 1M, 0.5M urea respectively and a final 4ml buffer A. The column was washed with 4ml of buffer B [20mM Tris-HCl (pH 8.5 at 4°C), 1M KCl, 5mM 2-mercaptoethanol, 10% glycerol] and a further 4ml of buffer A. Bound protein was eluted in 0.5ml fractions with buffer C [20mM Tris-HCl (pH 8.5 at 4°C), 100mM KCl, 5mM 2-mercaptoethanol, 10% glycerol, 100mM imidazole]. All fractions were analysed by SDS-PAGE to determine their protein content. Fractions containing purified Nef were subjected to protein quantification by Bradfords assay (appendix A13).

2.2.7 Immunoassays

Several polyclonal and monoclonal antibodies against Nef were available and the availability of my bacterially produced Nef allowed me to assess them for their potential reactivity to DU151 Nef. This would allow me to set up a viable immunoassay for determining the presence of Nef protein in the *Nicotiana benthamiana* based expression system. Table 2.1 below shows different antisera and mAbs tested in the laboratory. All commercially available antibodies were to the Nef of HIV-1 subtype B, which is prevalent in Europe from whence most of these reagents were imported.

Antibody name or source	specificity	Raised in
1.UKMRC	HIV-1 subtype B Nef monoclonal aa64-68	Balb C mouse
2.ARP 3107	HIV-1 subtype B Nef monoclonal aa71-90	Rat
3.LIIV-7	HIV-1 subtype B Nef polyclonal	Rabbit
4.Chemicon	HIV-1 subtype B C-terminal	Mouse
5.Clive Gray (NIV)	HIV-1 subtype B Nef Polyclonal	Sheep

Table 2.1: Anti Nef antibodies used in immunoassays

2.2.7a Western blotting

Western blots were carried out on purified protein after separation of proteins by a 15% SDS-PAGE. Immunoblotting was carried out according to the method in appendix A14. All 5 antisera were tested against Nef at dilutions in blocking buffer of 1:1000, 1:2000 and 1:5000. Secondary antibodies conjugated with alkaline phosphatase were added at dilutions in blocking buffer of 1: 5000 for goat anti rabbit, 1: 10000 for goat anti mouse and 1: 50000 for the mouse anti-sheep following the manufacturers (Sigma) recommendations. Blots were developed using NBT and BCIP.

2.2.7b ELISA

An initial pilot indirect enzyme linked immunosorbent assay (ELISA) (Barbara and Clark, 1982) (appendix A15) was carried out using the 5 antisera against bacterially expressed Nef. Anti-Nef antisera were diluted in PBS with Tween-20 in the following dilutions; 1:10, 1:100, 1:1000, 1:10000 and 1:100000. The corresponding alkaline phosphatase conjugated secondary antibodies were also diluted in PBS-Tween at the following dilutions; 1: 5000 for goat anti-rabbit, 1: 10000 for goat anti-mouse and 1: 50000 for the mouse anti-sheep. These were the recommended dilutions for western blots as specified by the manufacturers and are more concentrated than is usually required for ELISA. This ensured that secondary antibodies were well in excess so that any reaction will be easily discernible from background levels.

Having determined which antibodies were reactive, dilutions of primary antibodies of 1:1000, 1:5000 and 1:10000 and secondary antibody dilutions of 1:500, 1:1000 and 1:2000 were used to determine the optimal dilutions and ratios for detection of proteins by indirect ELISA.

2.3 RESULTS AND DISCUSSION

2.3.1 Amplification, subcloning and sequencing of *nef* gene

The *nef* gene was successfully amplified to give the expected fragment at 0.5kb as is shown in Figure 2.2. The strongest amplification was achieved using 3.5mM $MgCl_2$ in the reaction mix.

Figure 2.2: Gel electrophoresis of PCR products from amplification of *nef* with the addition of *Pac* 1 and *Xho* 1 restriction sites. The reaction was carried out with a magnesium titration in an effort to optimise the PCR. Lane 1, Marker: lane 2, 0.5mM $MgCl_2$: lane 3, 1.5mM $MgCl_2$: lane 4, 2.5mM $MgCl_2$: lane 5, 3.5mM $MgCl_2$: lane 6, no forward primer: lane 7, no reverse primer and lane 8, no template.

The correct incorporation of restriction sites *Pac* 1 and *Xho* 1 in the cloned PCR product was confirmed by digestion of extracted plasmid DNA from the clones with these two enzymes to excise the *nef* insert.

A majority of the screened clones contained the required restriction sites and could then be sequenced. Sequencing of those that did not have both sites would later show that in

some cases, the *Pac* 1 site was sometimes lost as an artifact of the PCR while the *Bam* H1 site internal to the *Pac* 1 site was always retained.

pGEMnef clone was checked by sequencing and the resulting sequence was aligned with the known DU151 sequence for comparison. The result of the two-sequence alignment is shown below

```
Fast alignment of DNA sequences DU151NEF.D and Gamanefx1

Ktuple=2  Gap_penalty=7

Upper line: DU151NEF.D, from 175 to 623
Lower line: pGEMnef, from 20 to 468

DU151NEF.D:Gamanefx1 identity= 100.00%(449/449) gap=0.00%(0/449)
175   CAAGCACAAGAGGAGGAACCAGAAGTAGGTTTTCCAGTCAGACCTCAGGTGCCTCTAAGA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
20    CAAGCACAAGAGGAGGAACCAGAAGTAGGTTTTCCAGTCAGACCTCAGGTGCCTCTAAGA

235   CCAATGACTTATAAGGCAGCATTCGATCTCAGCTTCTTTTTAAAAGAAAAGGGGGGACTG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
80    CCAATGACTTATAAGGCAGCATTCGATCTCAGCTTCTTTTTAAAAGAAAAGGGGGGACTG

295   GAAGGGTTAATTTACTCTAAGAAAAGACAAGACATTCTTGATTTGTGGGTCTATCACACA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
140   GAAGGGTTAATTTACTCTAAGAAAAGACAAGACATTCTTGATTTGTGGGTCTATCACACA

355   CAAGGCTACTTCCCTGATTGGCAAAACTACACACCGGGACCAGGGGTCAGACTTCCACTG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
200   CAAGGCTACTTCCCTGATTGGCAAAACTACACACCGGGACCAGGGGTCAGACTTCCACTG

415   ACCTTTGGATGGTGCTTCAAGCTAGTGCCAGTTGACCCAGAGGAAGTAGAAGAGGCCAAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
260   ACCTTTGGATGGTGCTTCAAGCTAGTGCCAGTTGACCCAGAGGAAGTAGAAGAGGCCAAC

475   AAACGACAAAACAACTGTTTGCTACACCCTTTGAGCCAGCATGGAATGGACGATGCAGAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
320   AAAGGAGAAAACAACTGTTTGCTACACCCTTTGAGCCAGCATGGAATGGAGGATGCAGAC

535   AGAGAAGTATTAAAGTGGGTGTTTGACAGCAGTCTACCACGCAGACACCTGGCCCGCGAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
380   AGAGAAGTATTAAAGTGGGTGTTTGACAGCAGTCTAGCACGCAGACACCTGGCCCGCGAG

595   AAACATCCGGAGTATTACAAAGACTGCTG
      ||||||||||||||||||||||||||||
440   AAACATCCGGAGTATTACAAAGACTGCTG
```

Alignment of the sequences showed 100% sequence identity.

2.3.2 Cloning of *nef* into pProEX™ HT

Restriction analysis of DNA resulting from ligation of pProEX HTb with the *nef* gene was done by digestion with *Bam* HI and *Xho* I. Digestion would excise the inserted *nef* gene to give a 0.5 kb band upon electrophoresis of the digested DNA on an agarose gel. This band was present in DNA from several clones. One clone was designated pProNef and used in all subsequent work.

2.3.3 Small Scale Induction of Nef Protein in *E. coli* Expression

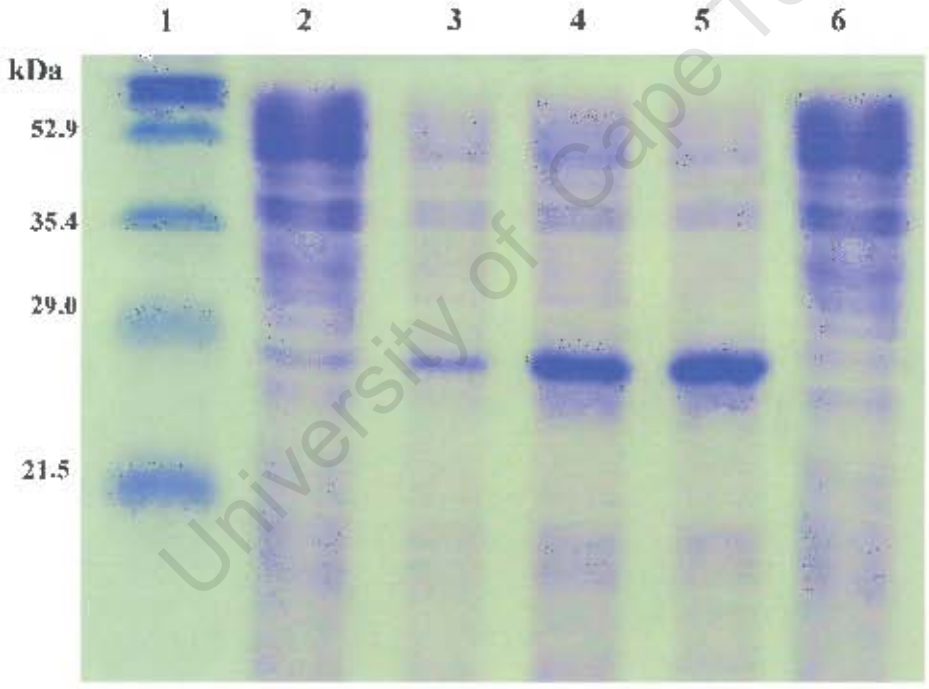


Figure 2.3: SDS-PAGE of total protein from a small-scale induction of pProNef. Lane 1, protein standards: Lane 2, uninduced *E. coli* harboring pProNef: Lane 3, *E. coli* harvested 1 hour after induction: Lane 4, *E. coli* harvested 2 hours after induction: Lane 5, *E. coli* harvested 3 hours after induction: Lane 6, induced *E. coli* with pProEX HTb.

Small scale induction of protein expression in pProNef harboring cells produced an increase in expression of a protein migrating just below the 28kDa protein standard (figure 2.3). This size is close to the predicted molecular weight of 25kDa for the product

of the cloned *nef* gene carrying a hexa-histidine tag (His(6)-Nef). Characterization of the band as His(6)-Nef was later confirmed by western blot (figure 2.7). From lane 2 in Figure 2.3 it can be seen that there is a low level of constitutive expression of the protein in the uninduced cells, where the His(6)-Nef band is not prominent. There is an increase in the expression of this protein at 1 hour after induction (lane 3) and the highest levels of expression of this protein appear after 2 and 3 hours (lanes 4 and 5), where the His(6)-Nef band is extremely prominent. No such band is generated from the induction of cells containing the pProEX HTb plasmid (lane 6 of figure 2.3). This result confirms expression of the *nef* gene in pProNef and demonstrates that the highest level of expression of the protein under the induction conditions used is achieved by induction for 3 hours.

2.3.4 Large Scale Induction of Nef Protein in *E. coli* Expression

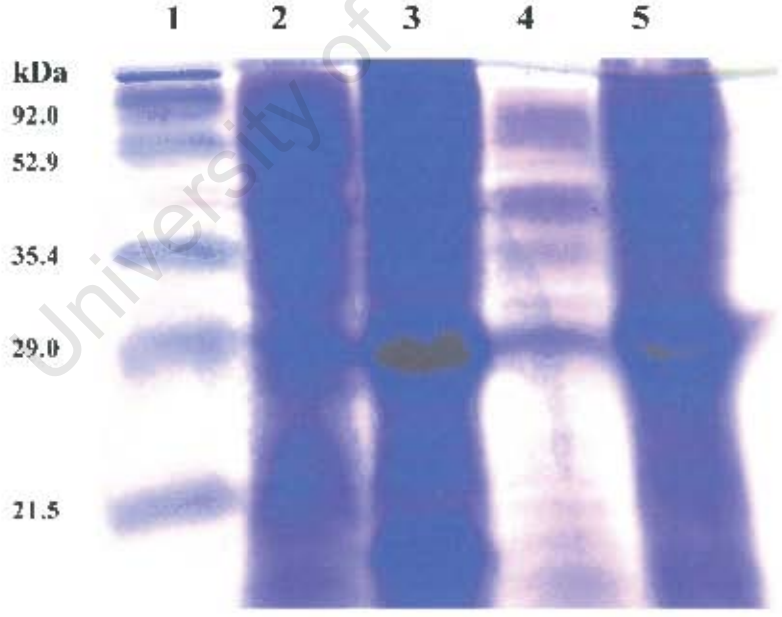


Figure 2.4: SDS-PAGE of pellet and supernatant fractions of lysed large scale induction cell preparations. Lane 1, Protein molecular weight standards: lane 2, total protein from uninduced cells: lane 3, total protein from induced cells: lane 4, Supernatant fraction from induced cells: lane 5, pellet from induced cells.

Based on the findings of the small scale induction, a large-scale induction of cells containing pProNef was carried for 3 hours. SDS-PAGE analysis (figure 2.4) showed the majority of the protein was present in the pellet fraction, after cell lysis (lane 5), while a small amount of the protein was found in the supernatant fraction (lane 4).

2.3.5 Purification of inclusion bodies

Inclusion bodies from cells induced with IPTG were isolated and solubilized. The SDS-PAGE in Figure 2.5 shows the resulting proteins. Note that the gel was run under visibly sub-optimal conditions due to the presence of urea and detergents in the protein solutions being resolved. The first step of the two step process removed all other cell debris and purified the inclusion bodies. The presence of the His(6)-Nef protein in the resulting pellet fraction is confirmed in lane 3. The second step was the solubilization which involved disrupting the purified bodies with urea. There was a band representing Nef in the soluble supernatant fraction obtained from this step in lane 4 indicating successful solubilization of the protein. In this state, the protein should be suitable for purification by affinity chromatography.

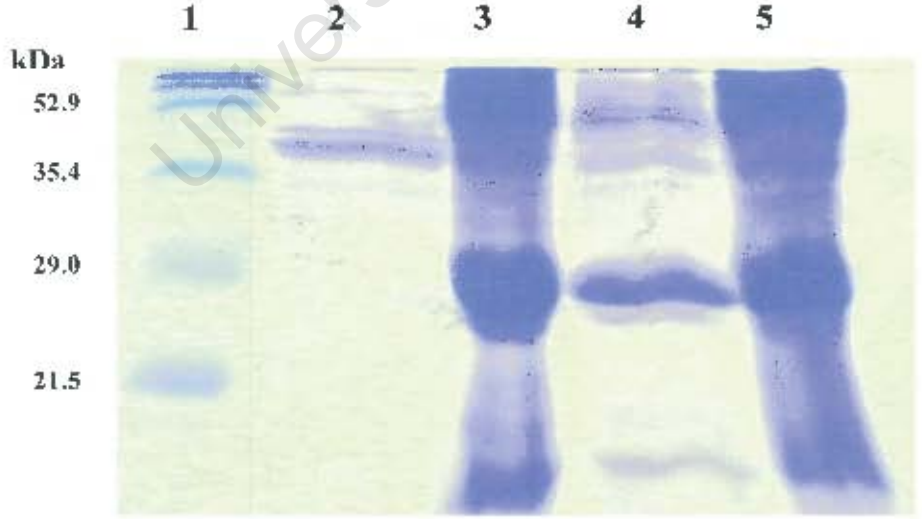


Figure 2.5: SDS-PAGE of all fractions resulting from purification and solubilisation of inclusion bodies. Lane 1, protein molecular weight standards: Lanes 2 &3, supernatant and pellet from inclusion body isolation: Lanes 4 & 5, pellet and supernatant from solubilisation of inclusion bodies.

2.3.6 Purification of Nef by Affinity Chromatography

Affinity chromatography to purify His(6)-Nef was carried out using a modified procedure to simultaneously refold the denatured protein (figure 2.6). The eluted fractions show highly purified protein without all of the other proteins present in the crude extract loaded onto the column. The purified protein was pooled and quantitated by Bradford's assay. The pooled protein was found to be at a concentration of about 4µg per ml. The protein was of high enough quality that it could have been used to produce polyclonal antibodies in rabbits against the HIV-1 Nef protein and for the optimization of ELISA techniques for detection of HIV-1 Nef expressed in other systems.

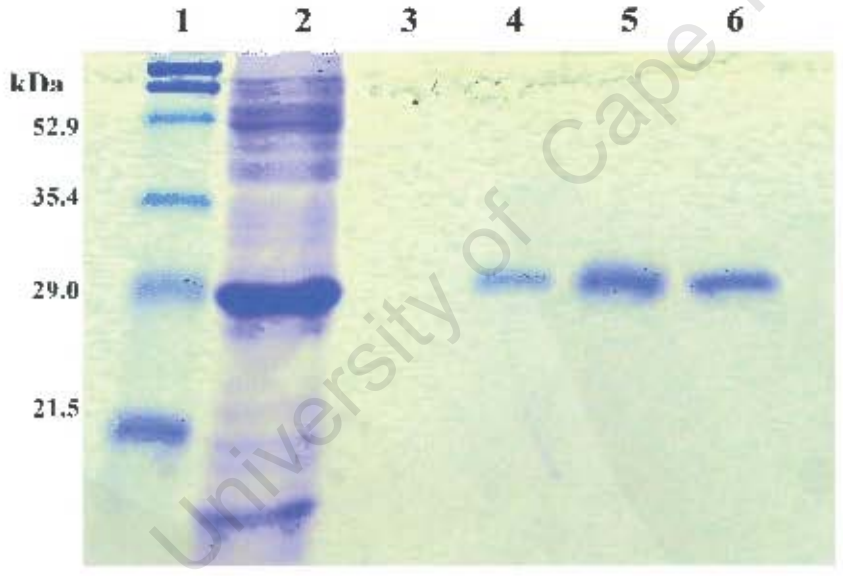


Figure 2.6. SDS-PAGE of elution fractions resulting from affinity chromatography conducted to purify Nef. Lane 1, protein molecular weight standards: Lane 2, crude protein loaded onto chromatography column: Lanes 3, 4, 5 & 6, eluted fractions from Ni-NTA column.

2.3.7: Immunoassays

2.2.7a Western blots

The bacterially expressed His(6)-Nef was produced as a very pure sample by affinity chromatograohy. This provided a potential reagent and positive control for other procedures. The identity of the 25kDa band produced by the pProNef clone could be confirmed by reaction with the anti-Nef antibodies available to us. In figure 2.7, five antisera were tested to the bacterially expressed His(6)-Nef to confirm its identity and establish which antiserum can be used in other procedures.

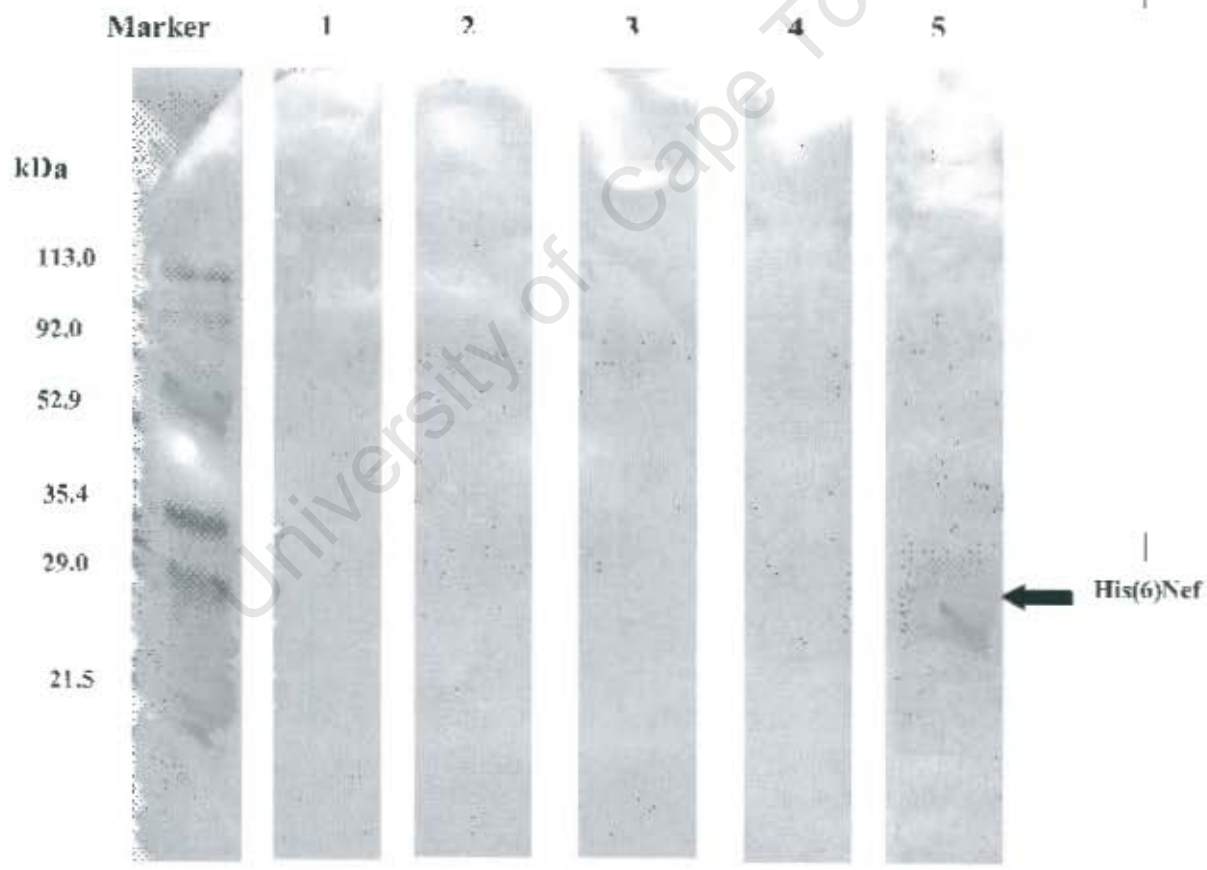


Figure 2.7: Western blots of the bacterially expressed Nef protein. Each strip was probed using a different antibody. Lane 1, UKMRC (monoclonal); lane 2, ARP 3107 (monoclonal); lane 3, LHV-7 (poly); lane 4, Chemicon (monoclonal) and lane 5, NIV (polyclonal).

Only the NIV sheep polyclonal antiserum produced a reaction with Nef showing a clear band at 25kDa where the His(6)-Nef band was present.

2.3.7b ELISA

In indirect ELISA reactions on purified His(6)-Nef using the five antibodies, only the NIV polyclonal antibody produced a positive serological reaction (figure 2.8). This antibody produced OD readings at 450nm of above 2.0 at the highest concentration of His(6)-Nef tested. None of the other antibodies produced any reaction above the background level, except the UK MRC monoclonal antibody. This antibody appeared to generate readings slightly above background OD readings but this was probably not significant as the error bars incorporates levels below the 0.5 level.

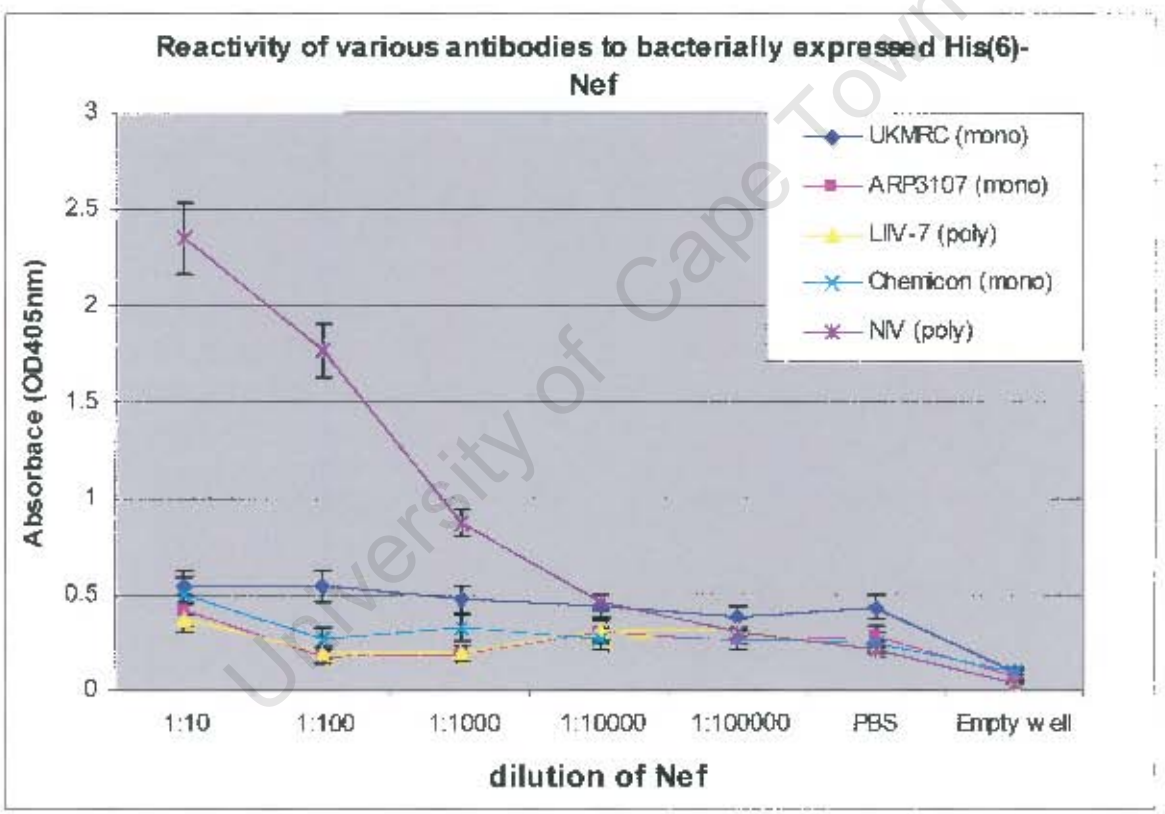


Figure 2.8: Results of ELISA reactions to test the reactivity of available antibodies to bacterially expressed His(6)-Nef.

Optimisation of the ELISA of Nef using the NIV sheep polyclonal anti Nef antiserum was carried out and the results in figure 2.9 show that a primary antibody at a dilution of 1 in 5000 and a secondary antibody at a dilution of 1 in 1000 gave the best signal and the

best ratio of positive signal to background. Of all the dilutions tested, these dilutions were the most optimal for use in the detection of Nef in *Nicotiana benthamiana* upon expression using the TMV-vector based expression system.

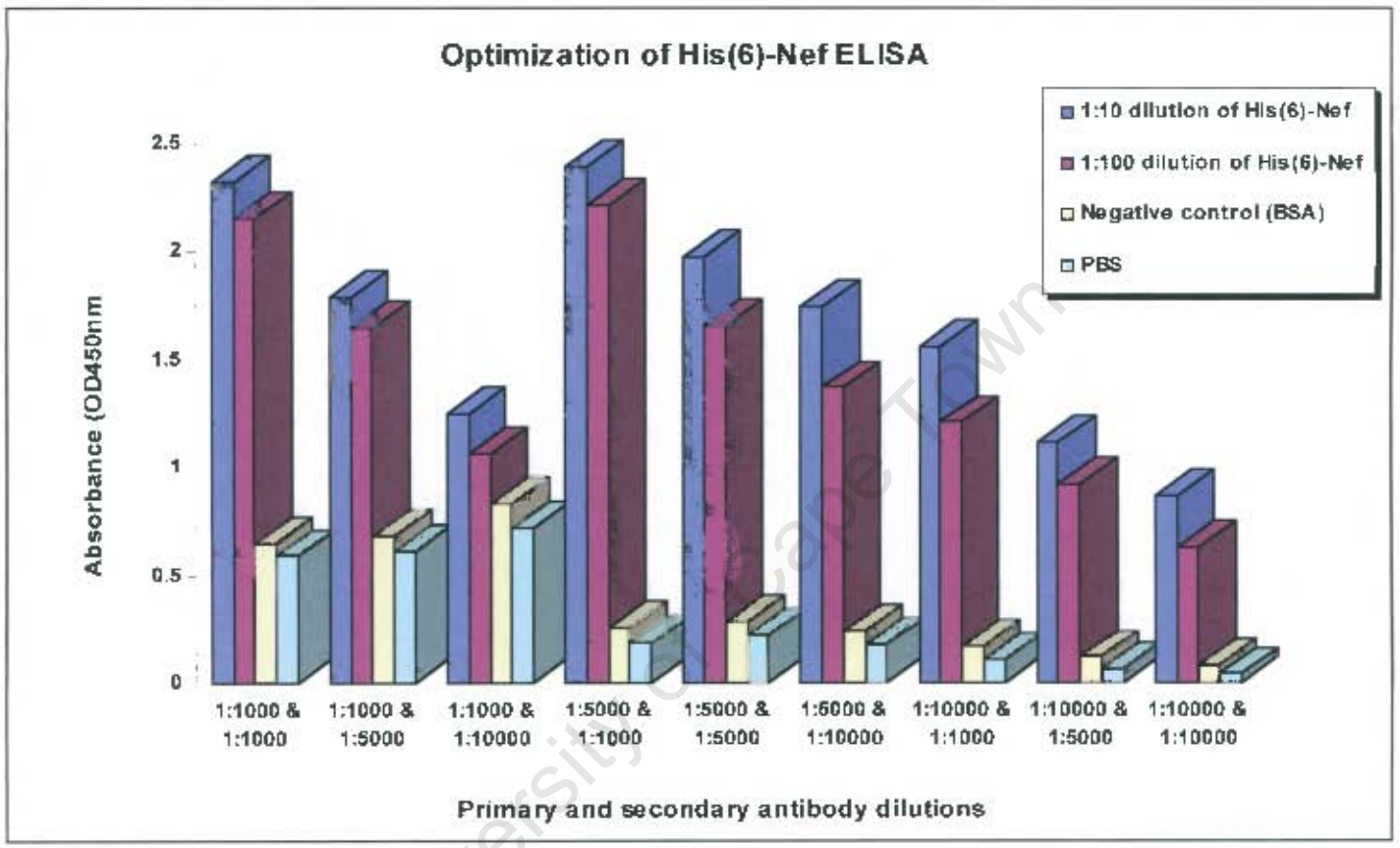


Figure 2.9: optimization of ELISA for Nef using the NIV sheep polyclonal anti-Nef antiserum.

2.4 CONCLUSIONS

The HIV-1 *nef* gene was successfully amplified from the cloned DU151 isolate DNA pDU151. Restriction sites for *Pac* 1, *BamH* 1 and *Xho* 1 were added and the first 59 codons were eliminated to address serious safety concerns that are associated with the cytotoxic effects of the N terminal region of Nef. The amplicon was examined once ligated into pGemT-Easy and grown up for screening. Clones of the amplicon in pGemT-Easy that showed the presence of the required enzyme sites were confirmed by sequencing to align perfectly with the known sequence of the DU151 *nef* gene.

The *nef* gene was excised using *BamH* 1 and *Xho* 1 and inserted into vector pProEx HTb to produce pProNef. A small scale induction of clones harboring this plasmid showed that the heterologous protein was well expressed upon induction with IPTG. This was observed by the increase in size of the band corresponding to His(6)-Nef at 2 and 3 hours after induction (figure 2.3). It was also established that expression peaked at 3 hours after induction and this informed the method of large scale induction.

Large scale induction for production purposes was carried out in 500ml volumes for 3 hours and the cells were harvested by centrifugation. The cells were treated with lysozyme and sonicated to harvest protein. The majority of the protein of interest remained in the insoluble fraction of the protein preparation, while only an insignificant amount of protein was to be found in the soluble fraction (figure 2.4). The strong Trc promoter leads to overproduction of His(6)-Nef in the cytoplasm. Such overproduction, along with unfamiliar host chaperones, is known to result in misfolding and segregation into insoluble inclusion bodies (Baneyx, 1999) and it is likely that this is what happened with His(6)-Nef. This necessitated an additional purification step in which inclusion bodies were separated and harvested and then solubilised for further purification. This situation may have been circumvented by reducing the level of induction through manipulations of temperature, optical density and time of induction. Recent work by Finzi *et al.* (2003) shows that large amounts of Nef become soluble when Nef-expressing bacteria are grown at 30 C (Finzi *et al.*, 2003). Modification of codon usage may also

contribute in optimization of expression. For example, the arginine codons AGA and AGG are rarely found in *E. coli* genes, the presence of such codons in cloned genes affects protein accumulation levels and mRNA and plasmid stability, in extreme cases it inhibits cell growth and hence protein synthesis (Zahn, 1996).

Purification and solubilization of inclusion bodies involved the use of the strong detergent Triton-X100 and urea at high concentrations. The fraction of soluble His(6)-Nef protein was increased in this process (Figure 2.5) with much of the host bacterial protein remaining insoluble. Affinity chromatography resulted in a very pure sample of the desired protein (Figure2.6). Two contaminant proteins are known to co-purify with His-tagged protein expressed in *E. coli* (Finzi *et al.*, 2003). The first is SlyD (GenBank database accession number P30856), a protein that was also referred to as wondrous histidine-rich protein (WHP) (Wulfing *et al.*, 1994). The second is GTP cyclohydrolase 1 (GCH1; GenBank database accession number P27511). Both proteins are rich in histidine or have some affinity for transitional metal ions making them likely to co-purify with His tagged proteins in IMAC. However, neither protein reacts with Nef-specific polyclonal antiserum (Finzi *et al.*, 2003) and thus should not present a problem in the development of immunoassays using His(6)-Nef. Should the need arise to remove contaminant proteins (e.g. for antibody production), the heparin binding properties of Nef can be exploited to get a very pure preparation of Nef (Kohleisen *et al.*, 1996).

In figure 2.6 it is clear that much of the protein is lost in the IMAC process. The concentration of His(6)-Nef in the eluted fractions was determined by Bradford's assay to be 4μg per ml. The capacity of the resin was sufficient to bind all the protein passed through and so loss of protein may have been due to insufficient binding to the histidine tag because much of the protein was still misfolded and the His tag may have been occluded from interaction with chelated nickel ions in the resin.

Western analysis of the protein using available antibodies confirms that it is indeed His(6)-Nef (figure 2.7). Of the five Nef-specific antisera tested, the only the NIV sheep polyclonal antiserum is reactive to the subtype C DU151 isolate encoded Nef. The lack of

reactivity with the monoclonal antibodies is most likely as a result of inter-subtype variation of the *nef* gene. All of the antisera used were obtained from Europe. They are all raised against the *nef* gene of HIV-1 subtype B which predominates in Europe. It is therefore unlikely that the monoclonal antibodies would have reacted to the subtype C Nef. Furthermore, because of the strong denaturing conditions used in the purification of His(6)-Nef, the conformational epitopes to which the monoclonal antibodies map would also not be present. This result underscores the importance of generating reagents specific to the local subtypes of HIV-1. Polyclonal antisera appear to have enough cross reactivity to allow widespread use in immunoassays. In at least two other studies involving Nef raised in mammalian cell culture, the same polyclonal antibody raised in sheep against GST-Nef has been the antibody of choice (Renkema *et al.*, 1999; Hiipakka *et al.*, 2001). It is not immediately clear why the other polyclonal antibody available (LIV-7) was also unreactive in both immunoassays. Although antibodies are generally stable and robust, is possible that poor storage could have led to degradation of this particular antibody.

As a final step, a dilution series of primary and secondary antibodies was tested in ELISA reactions of Nef (figure 2.9) and the optimal ratios were achieved with a primary sheep polyclonal anti HIV-1 Nef antibody at a dilution of 1 in 5000 and a secondary alkaline phosphatase conjugated mouse-anti-sheep monoclonal antibody at a dilution of 1 in 1000. Conditions for diagnostic ELISA reactions for the detection of Nef in the *Nicotiana benthamiana* based system would be informed by this result.

CHAPTER 3

Expression of Nef in *Nicotiana benthamiana* using a TMV based vector

3.1 INTRODUCTION

3.1.1 TMV

Tobacco mosaic virus (TMV) is a single stranded RNA virus and is the type member of the family *Tobamoviridae.* It is transmitted between *Nicotiana* plants through abrasions and breaks in the cell walls via direct physical contact. It is perhaps the best-studied virus infecting plants: in 1898 it became the first agent described as a virus (Beijerinck, 1898) and in 1982 it became the first plant virus genome to be sequenced (Goelet et al., 1982). Members of the group *Tobamoviridae* encapsidate a plus sense RNA genome of about 6.5kb into rods 300nm long and 18 nm in diameter, with a central hole of 4nm in diameter.

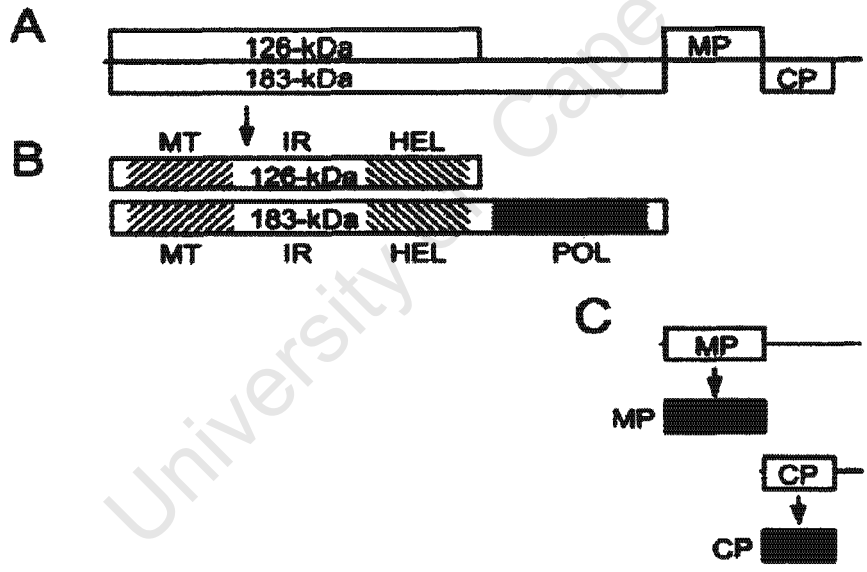


Figure 3.1: Schematic diagram of the TMV genome. (A) Organization of the TMV genomic RNA. Boxes represent open reading frames. Lines indicate non-translated sequences. (B) Schematic diagram of overlapping replication proteins. Shaded boxes represent domains similar with methyltransferases (MT), helicases (HEL), RNA dependent polymerases (POL). IR is the intervening region. (C) Subgenomic RNAs for the movement protein (MP) and capsid protein (CP) and their encoded proteins (Taken from Knapp *et al.*, 2001).

The TMV genome comprises a single positive sense RNA molecule 6395 nucleotides (nts) in length, containing four open reading frames (ORF) (fig 3.1) (Goelet *et al.*, 1982). The 5'-terminus of the TMV genomic RNA is capped with 7-methyl guanosine. The 5'-proximal ORFs that encode the overlapping 126 and 183 kDa replication proteins initiate at nt 69 and terminate with amber and ochre stop codons at nt 3417-3419 and nt 4917-1919, respectively (fig 3.1B). Both proteins are translated directly from the genomic RNA and are required for efficient replication (Ishikawa *et al.*, 1991). The 30 kDa movement protein (MP) (nt 4903-5709) and the 17.5 kDa capsid protein (CP) (nt 5712-6191) are expressed from individual 3'-co-terminal subgenomic mRNAs (sgRNAs) (fig 3.1C). A third sgRNA encoding a potential 54 kDa protein corresponding to the C-terminus of the 183 kDa protein (Sulzinski *et al.*, 1985) has been found to associate with polysomes in tobacco leaves (Zaitlin, 1999). The movement protein is required for cell to cell movement of the virus and associates with plasmodesmata, intercellular cytoplasmic channels, to modify their size exclusion limit (Wolf *et al.*, 1989). Additionally the MP cooperatively binds single stranded nucleic acids *in vitro* and interacts with plant cytoskeletal elements (Carrington *et al.*, 1996; Mas and Beachy, 2000). In addition to its coding function, the *mp* gene contains an origin of assembly sequence (OAS), from which assembly of viral RNA and coat protein into virus particles initiates (Zimmern, 1983), and a subgenomic promoter sequence that, in the natural virus genome, directs the synthesis of the mRNA encoding the coat protein (Grdzelishvili *et al.*, 2000). The 17.5kDa coat protein (CP) is produced late as the most abundant protein in infected cells. TMV RNA and CP aggregate spontaneously to form virions. Both the CP with its ability to assemble into virus particles and the OAS are involved in long distance movement and systemic spread of TMV and the virus particle is essential for such movement (Saito *et al.*, 1990). TMV particles are extremely stable and infectivity in plant sap survives heating to 90°C (Turpen and Reinl., 1998).

3.1.2: TMV based expression vector

One research area that distinguishes TMV from other plant viruses has been its widespread use to express foreign products in plants over the last decade. The ability to effectively manipulate RNA viruses through a cDNA copy allows for simpler cloning

strategies and increases their appeal as viral vectors. The cDNA copy can then be transcribed *in vitro* to produce infectious RNA molecules (Donson *et al.*, 1991). TMV vectors currently in use generally contain heterologous *cis*-acting elements and will tolerate the insertion of foreign sequences of useful lengths (Shivprasad *et al.*, 1999). Recently, a variety of foreign sequences have been expressed in plants, including whole proteins, peptides (Gilleland *et al.*, 2000; Nemichov *et al.*, 2000), RNA sequences to silence host genes (Kumagai *et al* 1995) and foreign proteins with pharmaceutical applications such as neomycin phosphotransferase and even whole antibodies and VLPs (Krebitz *et al.*, 2000; McCormick *et al.*, 1999; Wigdorovitz *et al.*, 1999). Monopartite TMV vectors are based on a design in which the foreign gene is generally inserted into the full length viral genome. One such TMV vector system has been constructed by Large Scale Biology Corporation., Vacaville CA.

The TMV-based vector pBSG1057 (figure 3.2) encodes the TMV-MP and CP of the TMV U5 strain also known as tobacco mild green mosaic virus (TMGMV) which is less virulent than the type member U1 strain and accumulates low levels of CP. The TMV vector contains genes encoding replication associated genes as well as a modified gene encoding the green fluorescent protein (GFP) of *Aequorea victoria*. Infection of tobacco plants with RNA transcripts of this vector result in high levels of expression of GFP. GFP expression is a marker of infection and can be visualised using UV light. Expression is accompanied by moderate curling of leaves and reduced growth, which are typical symptoms of TMV infection. Replacement of the *gfp* gene with a heterologous gene enables high-level expression of heterologous proteins in *Nicotiana benthamiana*. The pUC vector backbone with the selectable ampicillin resistance gene and the colE1 origin of replication is fused to the viral genome to enable genetic manipulation in E. coli. The system was first used to express enkephalin in inoculated tobacco (*Nicotiana tabacum)* and the kanamycin resistance gene in *Nicotiana benthamiana* in 1991 (Donson *et al.*, 1991).

In this chapter, the *gfp* gene was replaced with the *nef* encoding sequence in an attempt to express high levels of Nef in *Nicotiana benthamiana*. The expression of the *nef* gene is

tracked by simultaneously infecting control plants with pBSG1057 RNA transcripts. The spread of the TMV infection is monitored by checking for the appearance of GFP in the leaf tissues of the host by illumination with UV light. It is assumed that the infection of plants with the recombinant, *nef* expressing vector will spread and become systemic at a similar rate. It was hoped that this system may be used for cheap, large-scale production of reagents and possible HIV-1 antigens such as Nef.

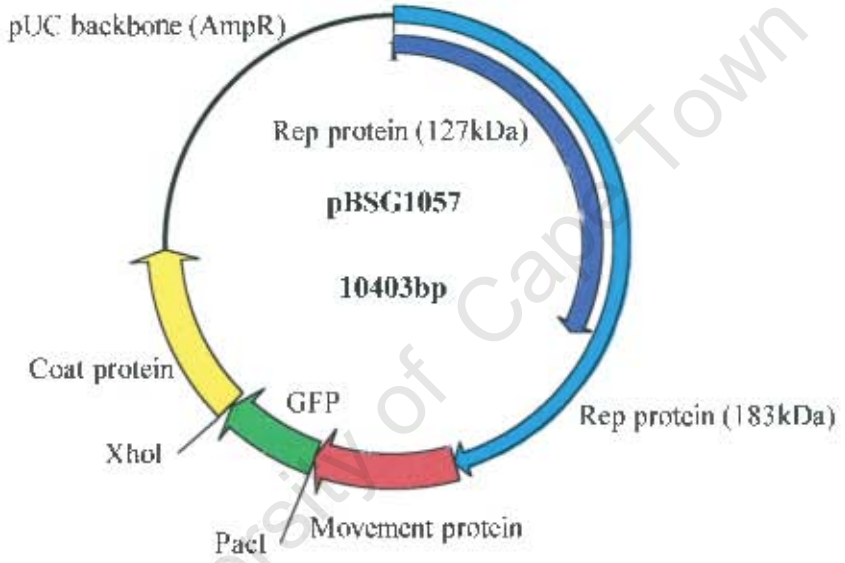


Figure 3.2: Plasmid map of TMV based expression vector pBSG1057

Production of proteins in plants can be viewed as a process similar to farming where characteristics of the plants will influence the yield. Under growth conditions where most environmental conditions are optimized and constant, the genetic variation in the crop species used becomes an increasingly central determinant of the eventual yield. *Nicotiana benthamiana* breeding lines have been developed over the years with increasingly specialized traits for research and other purposes. For our purposes, the permissiveness of the line, or its lack of resistance to infection, as well as its size at harvest and ontogenic characteristics are all important factors which will determine the eventual yield. Two

breeding lines of *Nicotiana benthamiana* were compared. One, designated LSB, was provided by Large Scale Biology and was developed to be particularly permissive for use in TMV vector related research. The other, designated UCT'95, was provided by the University of Cape Town and has also been used for research over the last few years.

The successful determination of permissiveness is dependent on the administration of an equal dose of inoculum to the two strains. Bawden and Price (1959) showed that although there is no obligate synergism between intact TMV particles as infectious agents, the infectious nucleic acid behaves differently and has an abnormally steep dilution curve. This has been evident in the reports of my colleagues who experience erratic infections using the transcribed pBSG1057 RNA. For the purpose of standardizing the infectious dose of recombinant TMV, dried leaf material from plants already infected with pBSG1057 transcripts and showing signs of systemic infection was homogenized and used as inoculum.

Due to low levels of expression with many genes introduced into plants, several methods have been employed in efforts to enhance expression (reviewed in chapter 1). Optimisation of codon usage has been shown to greatly enhance expression of heterologous proteins in plants. Rouwendal *et al* (1997) enhanced expression of the gfp gene from *Aequorea victoria* in transgenic tobacco by resynthesising the gene to adapt its codon usage for that of plants. It has also been demonstrated that chloroplast genes transferred to the nuclear genome have adjusted to nuclear base composition and codon usage (Oliver, 1990). In this work, I set out to compare total protein accumulated using a vector construct containing the wild type *nef* gene with that of one in which the gene was resynthesised to reflect plant codon bias.

3.2: MATERIALS AND METHODS

3.2.1 Comparison of plant host breeding lines

The vector pBSG1057 was obtained from Dr Kenneth Palmer at the Large Scale Biology Corporation in Vacaville California, USA. A large-scale plasmid DNA extraction of

pBSG1057 was carried out by the Nucleobond™ method. Production of RNA transcript of pBSG1057 and a luciferase gene under the control of a T7 RNA polymerase promoter as a control DNA was carried out using the RIBOMAX™ Large Scale RNA Production Kit (Promega) by the method specified by the manufacturer. Three week old *Nicotiana benthamiana* plants at the four leaf stage from the UCT'95 batch were used as initial host plants. The plants were mechanically inoculated using 50µl of the RNA reaction and 50µl of DEPC water. Inoculation was carried out using autoclaved cotton buds. The cotton buds were dipped in celite and then into the RNA solution and gently rubbed onto the leaves in such a way that the leaves were not visibly abraded. Plants were monitored daily for the presence of symptoms and the appearance of GFP. Visualisation of GFP was done in a dark room with a long wavelength (320nm) ultraviolet lamp. Once the infection had become systemic leaves were harvested and placed and sealed in universal 20ml bottles containing silica gel and desiccated for 3 months. About 500mg of dried leaf material was crushed and resuspended in 4ml PBS. This suspension was used for the inoculation of 10 plants from the LSB breeding line and 10 plants from the UCT'95 breeding line. Plants used in this experiment were 3 weeks old, were at or just past the four leaf stage of development and were selected for their uniformity, with all plants over 3cm in height and none having grown larger than 5cm. The plants were observed on a daily basis and all visible physiological and ontogenic changes were noted. Expression of GFP as an indicator of infection was monitored by illuminating plants with a long wavelength UV lamp.

3.2.2 Production of plant codon optimised *nef*

The HIV-1 subtype C *nef* gene from codons 60 to 207 was redesigned to reflect a plant codon bias based on the Nicotiana benthamiana codon usage. A Translation of the sequence of the subcloned DU151 *nef* gene was used to regenerate the *nef* gene based on the codon usage table of *Nicotiana benthamiana* provided by Genbank. This codon optimized *nef* gene (*conef*) was synthesized and delivered as the plasmid pGemconef with a *Pac* I and *Xho* I restriction site on the 3' and 5' end of the gene respectively for cloning into pBSG1057 (see figure 3.2). This plasmid was constructed by Geneart™ Inc (Germany).

3.2.3 Cloning of *nef* and *conef* into pBSG1057

0.5μg of pBSG1057 DNA was digested with 1 unit each of *Pac* I and *Xho* I. The digested DNA was electrophoresed in a 0.8 % TBE agarose gel with 0.5μg/ml ethidium bromide and the band containing the digested DNA excised from the gel and the cut plasmid DNA was purified and stored at 4°C. 0.5μg pG*nef* and 0.5μg pGemconef DNA were digested with 1 unit each of *Pac* I and *Xho* I. The digested DNA was electrophoresed in a 0.8 % TBE agarose gel and the bands containing the excised *nef* and *conef* DNA were purified from the gel.

The excised *nef* DNA and the *Pac* I/*Xho* I digested pBSG1057 DNA were ligated overnight at 4°C at a vector:insert ratio of 1:8. A similar reaction was set up with *conef* DNA and the digested pBSG1057 DNA. Ligated DNA was used to transform competent DH5α *E. coli*. Plasmid DNA from resulting colonies was extracted from overnight cultures by the miniprep method. The nature of the DNA was determined by restriction endonuclease digestion with *Pac* I and *Xho* I and agarose gel electophoresis. Clones of pBSG1057 containing *nef* DNA were designated pBSGNEF while those containing *conef* were designated pBSGCONEF.

3.2.4 *In vitro* transcription of recombinant TMV constructs

Large-scale plasmid DNA extractions of all pBSGNEF and pBSGCONEF were made using the Nucleobond™ kit. Production of RNA transcripts of pBSG1057, pBSGNEF and pBSGCONEF and a luciferase gene under the control of a T7 RNA polymerase promoter as a control DNA was carried out using the RIBOMAX™ Large Scale RNA Production Kit (Promega) by the method specified by the manufacturer. A total of 10μg of plasmid DNA template and 1μg of control DNA was used in the reactions. Results of the *invitro* transcription were determined by electrophoresis in a 0.8 % TBE agarose gel with 0.5μg/ml ethidium bromide at 100V for 20 minutes. 1μl of transcribed RNA was loaded on the gel and a total of 300ng of untranscribed plasmid DNA was also loaded as a control.

3.2.5 Mechanical infection of *Nicotiana benthamiana*

Three week old *Nicotiana benthamiana* plants at the four leaf stage from the UCT'95 breeding line were used as host plants. The plants were mechanically inoculated using 50µl of the *invitro* transcription reactions and 50µl of DEPC water. Inoculation was carried out using autoclaved cotton buds. The cotton buds were dipped in celite and then into the RNA solution and gently rubbed onto the leaves in such a way that the leaf surface was not visibly abraded. Initial inoculations were carried out in duplicate with two plants inoculated with pBSG1057, pBSGNEF and pBSGCONEF respectively and two plants with water as a negative control. Plants were monitored daily for the presence of symptoms and the appearance of GFP. Visualisation of GFP was done in a dark room with a long wavelength ultraviolet lamp. Once the infection had become systemic leaves were harvested and subjected to tests for the presence of the desired RNA and protein.

3.2.6 RNA extraction, reverse transcription and PCR amplification of cDNA

The upper leaves of recombinant TMV infected plants were cut from infected and control plants and care was taken not to include the inoculated leaves as part of the sample in order to eliminate the possibility of residual inoculated RNA reacting in the reverse transcription and PCR steps that followed RNA extraction. 100mg of leaf material from a harvested plant was placed in a mortar and homogenized in liquid N_2. A portion of the homogenized leaves was used to extract RNA while the rest was used for protein analysis. RNA from leaf material was extracted into TRIZOL (Appendix A10).

RNA extracted from each plant was used to conduct reverse transcription and PCR amplification of the resulting cDNA in a single step (Appendix A11). C. therm™ DNA polymerase™ (Roche™) has magnesium dependent reverse transcriptase activity allowing reverse transcription and polymerase chain reaction to be performed in a single reaction mixture when used in conjunction with Taq DNA polymerase ™ in what is termed a one step RT-PCR. Primers used were appropriate to prime the reverse transcription reaction as well as the PCR reaction that followed. As a positive control for

the RT-PCR reaction, RNA extracted from the pBSG1057 infected plants was subjected to RTPCR using an internal *gfp* primer set.

The following primer sets were used.

For pBSGNEF:

Forward primer: 5'-CCTTAATTAAGGATCCATGCAAGCACAAGAGGAGGAAC-3'

Reverse primer: 5'-CGGCTCGAGTCAGCAGTCTTTGTAATACTCC-3'

For pBSGCONEF:

Forward primer: 5'-ATCTCGCGAATGCTTCAAGCTCAAGAAG-3'

Reverse primer: 5'-TCCTCGAGACAATCCTTATAATATTCTGG-3'

For pBSG1057:

Forward primer: 5'-GGTTTAATTAAAATGGAGCCAGTAGATCCTAG-3'

Reverse primer: 5'-GGATCTCGAGGATATCCTCCACCTTCTTCTTCG-3'

3.2.7 Detection of proteins in harvested leaves

Homogenized leaf material from plants infected with each construct which was not used in RNA extraction was used for detection of proteins by SDS-PAGE and Western blot. This homogenized leaf material was suspended in 0.5ml of PBS and vortexed. The homogenate was centrifuged for a minute to remove large particulate matter and the supernatant plant sap extract was transferred to a fresh tube. The plant sap was placed in SDS-PAGE loading buffer and incubated at 80 C for 10 minutes. 10µl of this was then resolved a 15% SDS-PAGE gel. For concentration of plant sap 10g of leaves from a fresh batch of plants infected with pBSGNEF and pBSGCONEF respectively were homogenized in a food processor. The homogenate was suspended in PBS to a final volume of 10.5ml and centrifuged at 3000x g for 5 minutes to pellet down the insoluble leaf material. 5ml of sap was removed from each homogenate.

3.2.8 Concentration of plant leaf extract

Concentration of plant sap was achieved by ultrafiltration of the plant sap through an anisotropic membrane. Centrifugal force drives solvents of low molecular weight solutes through the membrane into a filtrate vial. The membrane support contains a circular

outlet with ducts for solvent flow (Figure 3.3). Retained microsolutes are above the membrane inside the sample reservoir at a greatly reduced volume, while the filtrate will contain microsolutes of molecular weight below the cutoff value of the membrane. A two step process was used to concentrate all the proteins between 30 and 10 kDa in molecular weight.

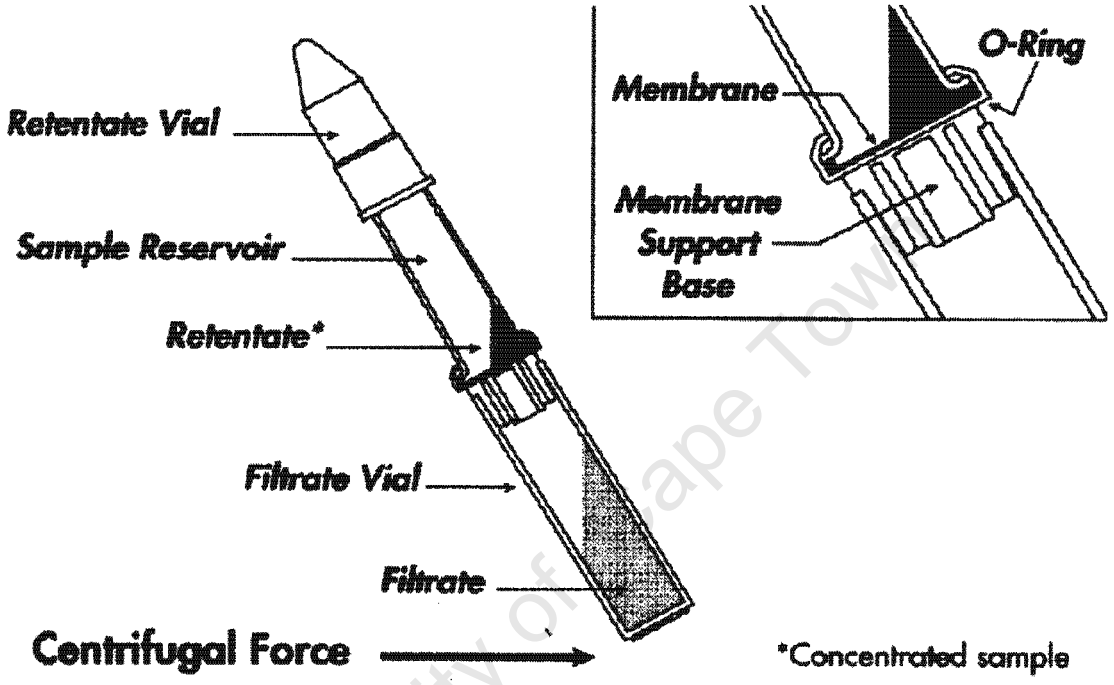


Figure 3.3. The Millipore Centricon ® centrifugal filter device shown in its configuration during the filtering process. The retentate is recovered by inverting the device and centrifuging in the retentate into the retentate vial (taken from Centricon filter device user's manual 99259, Rev. V 11/01).

In the first step, a Centricon® YM-30 centrifugal filter device with a 30000 molecular weight cut off was used. Samples containing sap from pBSGNEF and pBSGCONEF infected plants in PBS were added to the sample reservoir (2ml maximum volume). The devices were sealed by attaching a retentate vial to the top of the sample reservoir. The covered devices were the attatched to a filtrate vial and placed in a fixed angle centrifuge. The Centricon YM-30 centrifugal devices were centrifuged at 5000 x g for an hour after which more sample was added to the sample reservoirs and the centrifugation repeated. A total of 5ml of plant sap per device was filtered. The filtrates containing proteins smaller than 30kDa were then collected and recentrifuged using Centricon® YM-10 devices.

Centrifugation was carried out at a speed of 5000 x g for an hour after which more sample added to each tube. Filtration was carried out until all the sample had passed through the membrane. The retentate was recovered by placing a retentate vial over the sample reservoir and inverting the unit. The units were then centrifuged at 3000 x g for 3 minutes to transfer the concentrates into the vials. A total of 200µl of concentrate was recovered per device. The recovered retentates were then subjected to tests by Western blot and ELISA.

3.2.9: Immunoassays

3.2.9a: Western blotting

Proteins in plant sap samples were resolved by SDS-PAGE using a 15% gel. Resolved proteins were transferred onto nitrocellulose immunoassayed by the western blotting method detailed in appendix A14. The polyclonal antisera tested against the plant sap are listed below in table 3.1. Gels were run in duplicate with only one of the gels used for blotting while the other was stained with coamassie blue for visualisation of the proteins.

Primary antibody	Raised in	Source	Dilution	Secondary antibody	Source	Dilution
Anti-TMV	Rabbit	MCB Dept UCT	1:1000	Goat anti rabbit	SIGMA	1:5000
Anti-Nef	Sheep	NIV	1:5000	Mouse anti sheep	SIGMA	1:50000

Table 3.1: Polyclonal antisera used in western blots on plant extract

3.2.9b: Indirect ELISA

Plant sap from harvested leaves and concentrated plant sap were each subjected to indirect ELISA (Appendix A15) using the polyclonal anti-Nef antiserum raised in sheep. Reactions of each dilution of 1:10, 1:100, 1:1000 and 1:10000 were done in triplicate on

adjacent wells on the ELISA plate for extracts of plants infected with pBSG1057, pBSGNEF, pBSGCONEF and a healthy plant, concentrated extract from plants infected with pBSG1057, pBSGNEF, pBSGCONEF and a healthy plant with bacterially expressed His(6)-Nef used as a positive standard. Results were taken from the mean value of the three reactions of each sample.

3.2.10 Quantitation of Nef by western blot

Quantitation of a protein by western blot first requires the establishment of the dynamic working range (DWR) for the particular method and assay conditions. The DWR is the range in an assay where the immunolabeling slope increases significantly with increasing protein load. In western blots, samples with very low or very high immunoreactive signals are typically outside of the DWR and such samples cannot be reliably quantified in the blot.

Upper limit of DWR

Wells of a polyacrylamide gel were loaded with increasing amounts of bacterially expressed His(6)-Nef in the following order; 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.5 and 5.0μg respectively. The proteins were resolved on the gel and transferred to a nitrocellulose membrane. The blot was then probed with anti Nef antiserum at the established optimal dilution of 1:5000. Membrane from the developed western blot was visually captured by the Genegenius® bio-imaging system and subjected to manual densitometric readings using the GeneTools version 3.00.13 program from Syngene®. Arbitrary densitometric units for the immunolabeled bands were determined and plotted versus total protein loaded per lane (in μg). The upper limit of the DWR was established as that level of protein at which the signal saturates and the immunolabeling signal begins to plateau and fluctuate. Quantification above this region is unreliable.

Lower limit of DWR

Wells of a SDS-PAGE were loaded with increasing amounts of bacterially expressed His(6)-Nef in the following order; 0.0125, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5μg

respectively. The proteins were resolved on the gel and transferred to a nitrocellulose membrane. The blot was then probed with anti Nef antiserum at the established optimal dilution of 1:5000. Membrane from the developed western blot was visually captured by the Genegenius® bio imaging system and subjected to manual densitometric readings using the GeneTools version 3.00.13 program from Syngene®. Arbitrary densitometric units for the immunolabeled bands were determined and plotted versus total protein loaded per lane (in μg). The level at which the signal becomes unreliably weak and unquantifiable is the lower limit of the DWR.

Quantitation of Nef in concentrated plant sap samples

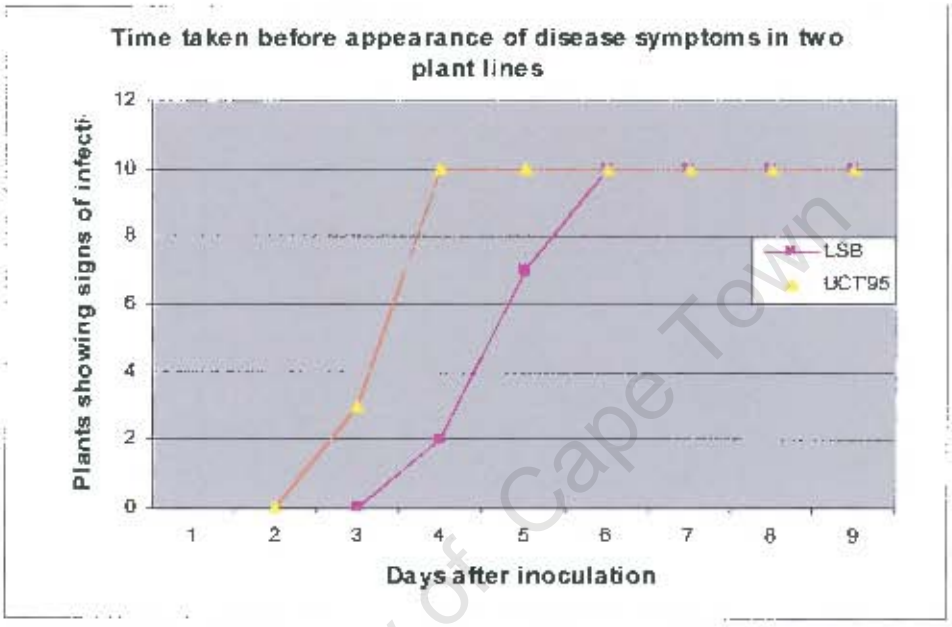
Once the DWR was determined, a standard curve was run on the same gel as the samples of interest, and quantitation of the levels of Nef was made by interpolating the signal from the sample within the DWR of the standard curve. The standard curve was made by loading wells with 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0μg of His(6)-Nef respectively and loading adjacent wells with 30μl of concentrated plant sap from pBSGNEF and pBSGCONEF infected plants. These volumes ensured that plant produced Nef protein levels fall within the DWR. All blots were carried out in duplicate and the average of the two quantities calculated from each standard curve was used. In order to establish the reproducibility in the result of the quantitation of Nef, the process of infection of plants with pBSGNEF and pBSGCONEF, harvesting and concentration of leaf material and quantitation of Nef by western blot was repeated twice more. Because of variations in transfer of proteins to the blotting membrane and other possible variables, a new standard was run for each quantitation.

3.3: RESULTS AND DISCUSSION

3.3.1 Comparison of plant host breeding lines

The results (figure 3.4A) show that initial fluorescent spots were visible on plants from the UCT'95 batch 2 days after inoculation with RNA while the LSB batch showed these initial signs of infection after only 3 days post-inoculation. The infection progressed at a

A)



B)

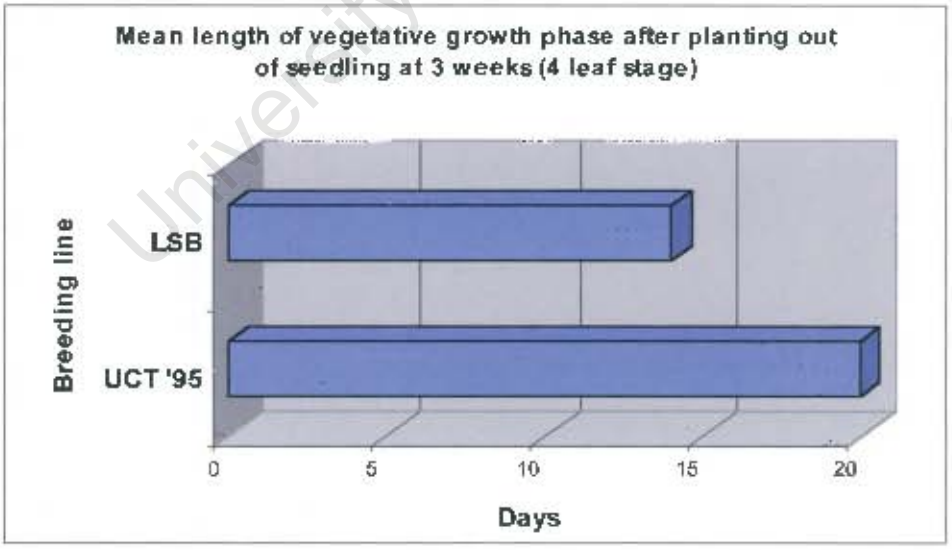


Figure 3.4: Graphs showing differences in A) time taken before appearance of disease symptoms and B) mean length of vegetative growth phase between LSB and UCT'95 strains of *Nicotiana benthamiana*.

Figure 3.5: Healthy *Nicotiana benthamiana* plants from the LSB (left) and UCT1995 (right) plant lines at harvest time 14 days after the inoculation date.

faster rate in the UCT'95 batch with all plants showing signs of infection by day 4 while in the LSB line, all plants showed infection at day 6.

Long distance movement of the virus, which was manifested by the presence of fluorescent spots in the apical meristem, occurred more rapidly in the UCT'95 line than in the LSB line. As such, the plants in the UCT'95 line were ready for harvesting at 7 days post-inoculation while those in the LSB batch were ready after 9 days. Figure 3.4B tracks the main ontogenic changes occurring in the two breeding lines in terms of the length of the vegetative growth period which is concluded by the onset of flowering. The result shows that in the plants from LSB plant line there were visible signs of the initiation of flowering after 14 days. In the UCT'95 plant line there are signs of flowering after 20 days post inoculation. The change of an apex from vegetative to the flowering state represents the outcome of an internal autonomous progression which is modulated to varying degrees, depending on the cultivar, by low temperature and photoperiod (Milthorpe and Moorby, 1979). In the presence of uniform temperatures and photoperiod the more rapid progression towards flowering of plants from the LSB batch can be attributed to a genetic predisposition to do so.

Figure 3.5 shows the appearance of the plants at harvest time. Clear differences in the ontology of the plants are visible with the UCT'95 plant line having a higher number of larger and broader leaves. This breeding line is also still in its vegetative growth stage while the LSB plant line is well into its flowering stage of growth. As a result, the amount of biomass generated per plant by the UCT'95 plant line was on average 30% higher than that of the LSB batch.

3.3.2 Production of plant codon optimised *nef*

The HIV-1 subtype C *nef* gene was re-synthesized by Geneart™ and delivered as plasmid pGemconef. The new sequence shared 75.82% identity with the wild type gene. The sequence below shows the modified DNA sequence of the plant codon optimized *nef* gene with the expected translation.

```
Translation of Nefopt(1-468)
Universal code
Total amino acid number: 156, MW=18200
Max ORF: 1-468, 156 AA, MW=18200

1          ATGTTGGCGAATGCTTCAAGCTCAAGAAGAAGAACCAGAAGTTGGTTTTCCAGTTAGACCA
1                    M  L  Q  A  Q  E  E  E  P  E  V  G  F  P  V  R  P

61         CAAGTTCCACTTAGACCAATGACTTATAAGGCTGCTTTTGATCTTTCTTTTTTTCTTAAG
21          Q  V  P  L  R  P  M  T  Y  K  A  A  F  D  L  S  F  F  L  K

121        GAAAAGGGTGGTCTTGAAGGTCTTATTTATTCTAAGAAGAGACAAGATATTCTTGATCTT
41          E  K  G  G  L  E  G  L  I  Y  S  K  K  R  Q  D  I  L  D  L

181        TGGGTTTATGGTACTCAAGGTTATTTTCCAGATTGGCAAAATTATACTCCAGGTCCAGGT
61          W  V  Y  G  T  Q  G  Y  F  P  D  W  Q  N  Y  T  P  G  P  G

241        GTTAGACTTCCACTTACTTTTGGTTGGTGTTTTAAGCTTGTTCCAGTTGATCCAGAAGAA
81          V  R  L  P  L  T  F  G  W  C  F  K  L  V  P  V  D  P  E  E

301        GTTGAAGAAGCTAATAAGGGTGAAAATAATTGTCTTCTTCATCCACTTTCTCAACATGGT
101         V  E  E  A  N  K  G  E  N  N  C  L  L  H  P  L  S  Q  H  G

361        ATGGAAGATGCTGATAGAGAAGTTCTTAAGTGGGTTTTTGATTCTTCTCTTGCTAGAAGA
121         M  E  D  A  D  R  E  V  L  K  W  V  F  D  S  S  L  A  R  R

421        CATCTTGCTAGAGAAAAGCATCCAGAATATTATAAGGATTGTCTCGAG
141         H  L  A  R  E  K  H  P  E  Y  Y  K  D  C
```

3.3.3 Cloning of *nef* and *conef* into pBSG1057

DNA from pGemnef and pGemconef was succesfully used to construct pBSGNEF and pBSGCONEF. The succesful introduction of the *nef* and *conef* genes into pBSG1057 as a replacement of *gfp* was confirmed by endonuclease digestion. The plasmids pBSGNEF and pBSGCONEF are digested with *Pac* 1 and *Xho* 1 to excise the inserted *nef* and *conef* genes respectively and the presence of 500bp bands on the gel after digestion indicated that correctly sized fragments had been released, corresponding to the expected size of the *nef* and *conef* fragments.

3.3.4 *In vitro* transcription of constructs

In vitro transcription products were detected on a 0.8% agarose TBE gel (Figure 3.6). This check showed the presence of sufficient RNA transcript from the rTMV constructs to initiate an infection in *Nicotiana benthamiana*. The RNA appears in the form of a smear for pBSG1057 (lane 3), pBSGNEF (lane 4), pBSGCONEF (lane 5) due to degradation that occurs during electrophoresis. The smaller control luciferase gene generates a larger quantity of RNA because of its smaller size and its more specific T7 RNA polymerase promoter. Untranscribed pBSG1057 plasmid DNA is seen in lane 2.

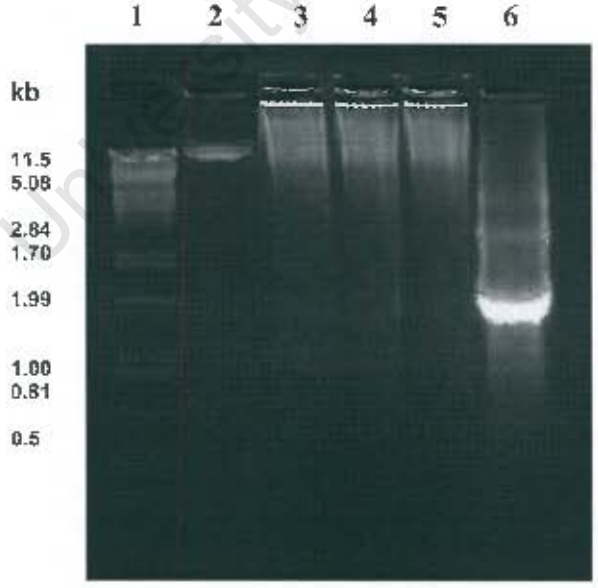


Figure 3.6: Agarose gel showing *in vitro* transcripts of plasmids. Lane 1, λ/Pst 1 DNA marker; lane 2, pBSG1057 plasmid DNA; lane 3, pBSG1057 transcription; lane 4, pBSGNEF transcription; lane 5, pBSGCONEF transcription and lane 6, control plasmid transcription.

3.3.5 Mechanical infection of *Nicotiana benthamiana*

Mechanical inoculation of plants resulted in successful infection and expression of GFP after several days in the pBSG1057 infected control plants. The presence of infection in plants inoculated with pBSGNEF and pBSGCONEF was confirmed by the presence of mild symptoms a week after inoculation.

3.3.6 RNA Extraction, reverse transcription and PCR amplification of cDNA

RT-PCR of RNA from plants infected with the two constructs yielded amplified DNA

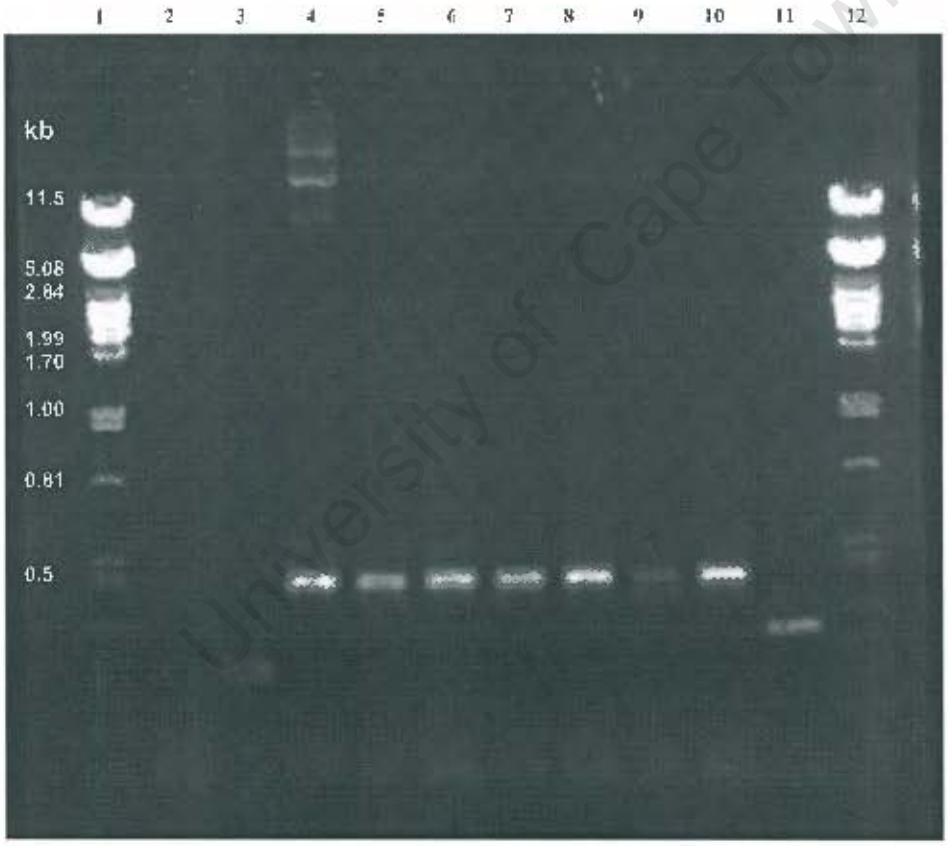


Figure 3.7: RT-PCR of RNA extracted from plants. Lane 1, λPst1 marker; lane2, pBSGNEF and pBSGCONEF infected plant RNA with all primers and RT step omitted; lane 3, pBSG1057 plasmid with gfp primers; lane 4, pBSGNEF plasmid DNA with nef primers; lanes 5 and 6, pBSGNEF infected plant RNA with nef primers; lane 7, pBSGCONEF plasmid DNA with conef primers; lanes 8 to 10, pBSGCONEF infected plant RNA with conef primers; lane 11, pBSG1057 infected plant RNA with gfp primers and lane 12, λPst1 marker

fragments at the expected size of 450bp indicating the presence of *nef* and *conef* mRNA. As can be seen in lane 2 (figure 3.7) where all the constructs and primers were included but the reverse transcription step was omitted, there was no priming up of DNA indicating that there was no residual DNA from the inoculation of plants. Both positive controls where the reaction was carried out with plasmid DNA gave the expected reaction. In lane 11, *gfp* RNA amplified with two internal primers to give a band of about 200bp.

3.3.7 Analysis of plant extract for Nef

3.3.7a SDS-PAGE and western blot analysis

SDS-PAGE analysis of plant extract (figure 3.8A) showed the presence of rTMV infection by the presence of a TMV coat protein band at about 22kDa in lanes 5 and 6. This was the only significant protein under 30kDa visible by staining of resolved unconcentrated proteins with Coomassie blue on the gel. Western blot probed with anti-TMV antiserum confirms the identity of this band in lanes B5 and 6. Concentration of the sap leads to an increase in the amount of TMV coat protein that is visible in the gel (lanes A2 and A3). Dimerisation of the TMV coat protein particles under high concentration produces a second band which runs at about 35kDa and this is seen in lanes A2 and A3. Both large bands react when probed with anti-TMV antiserum in lanes B2 and B3. Concentration of the plant sap produced another band at 21kDa (lanes A2 and A3) which reacted when probed with anti-Nef antiserum in lane C2. There was also a reaction with the bacterially expressed His(6)-Nef positive control in lane C8. There was no reaction in the lanes loaded with healthy plant sap tested against either the anti-Nef (C5 and C6) or anti-TMV (B5 and B6) antisera. The control samples of both the plant extract (lanes B7 and C7) as well as the concentrated healthy plant extract (lanes B4 and C4) were all unreactive in western blots.

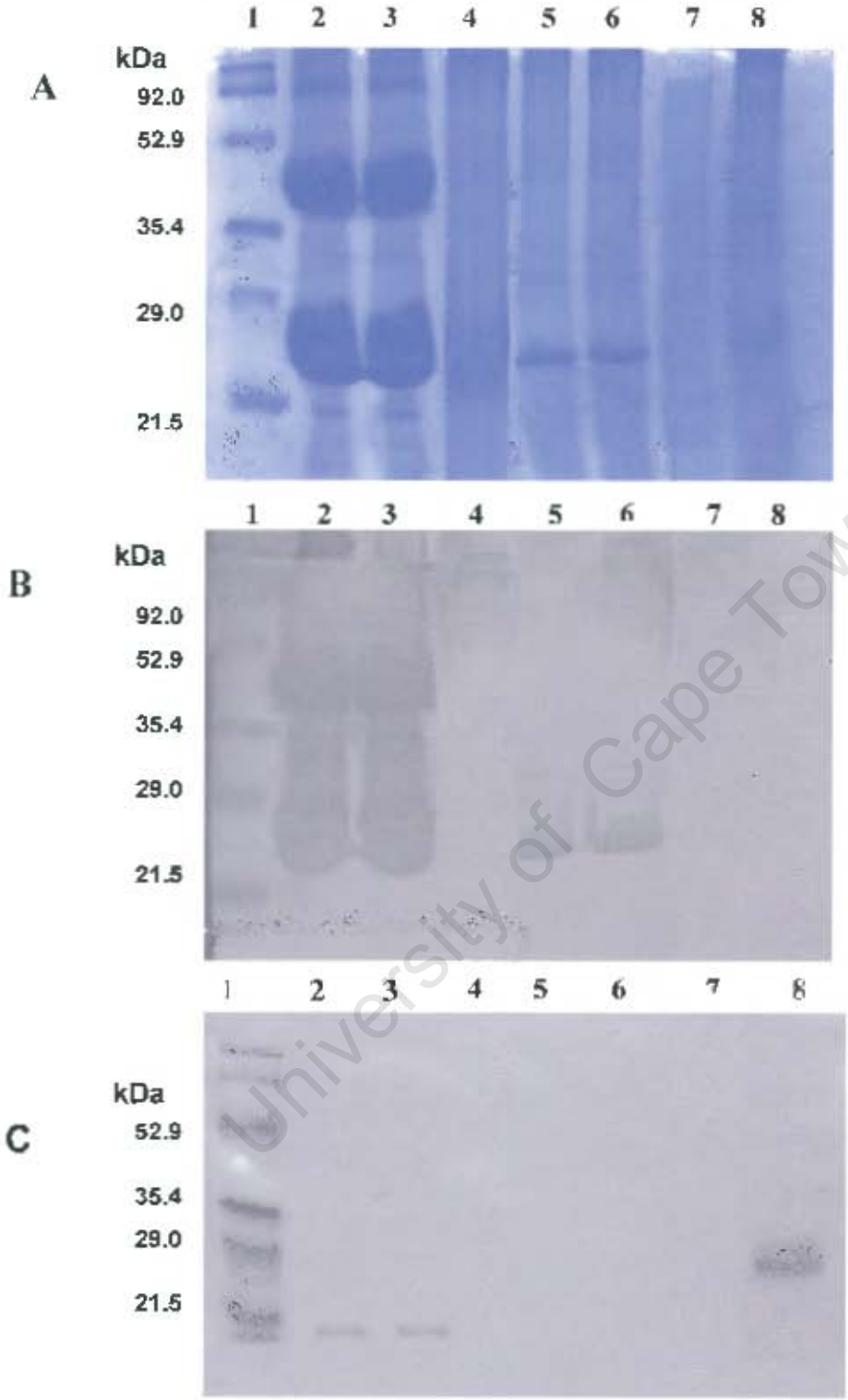


Figure 3.8: Analysis of plant extract of plants infected with rTMV RNA. A) Coomassie stained SDS PAGE of plant sap. B) Western blot probed with polyclonal anti TMV antiserum. C) Western blot probed with polyclonal anti HIV-1 Nef antiserum. In all cases Lane 1, prestained low molecular weight protein marker; lane 2, pBSGNEF filter concentrated plant sap; lane 3, pBSGCONEF filter concentrated sap; lane 4, filter concentrated healthy plant extract; lane 5 pBSGNEF plant extract; lane 6, pBSGCONEF plant extract; Lane 7, healthy plant extract and Lane 8, bacterially expressed Nef.

3.3.7b Indirect ELISA

Indirect ELISA carried out on plant extract of plants infected with pBSGNEF and pBSGCONEF (Figure 3.9) showed weak but significant serological reaction at a 1:10 dilution. A 1:100 dilution of plant extract produced a reaction only slightly above the background level resulting from absorbance readings of wells loaded with PBS. The reaction of the pBSGCONEF plant extract was stronger than that of the pBSGNEF plant. The serological reactions were increased in the concentrated plant extracts of both the pBSGCONEF and pBSGNEF with the former again producing a stronger reaction. The concentrated healthy plant sap reacted more strongly than the unconcentrated sap but both remained near background levels. The bacterially expressed His(6)-Nef was a positive control for the reaction and produced the strongest positive reaction at all dilutions.

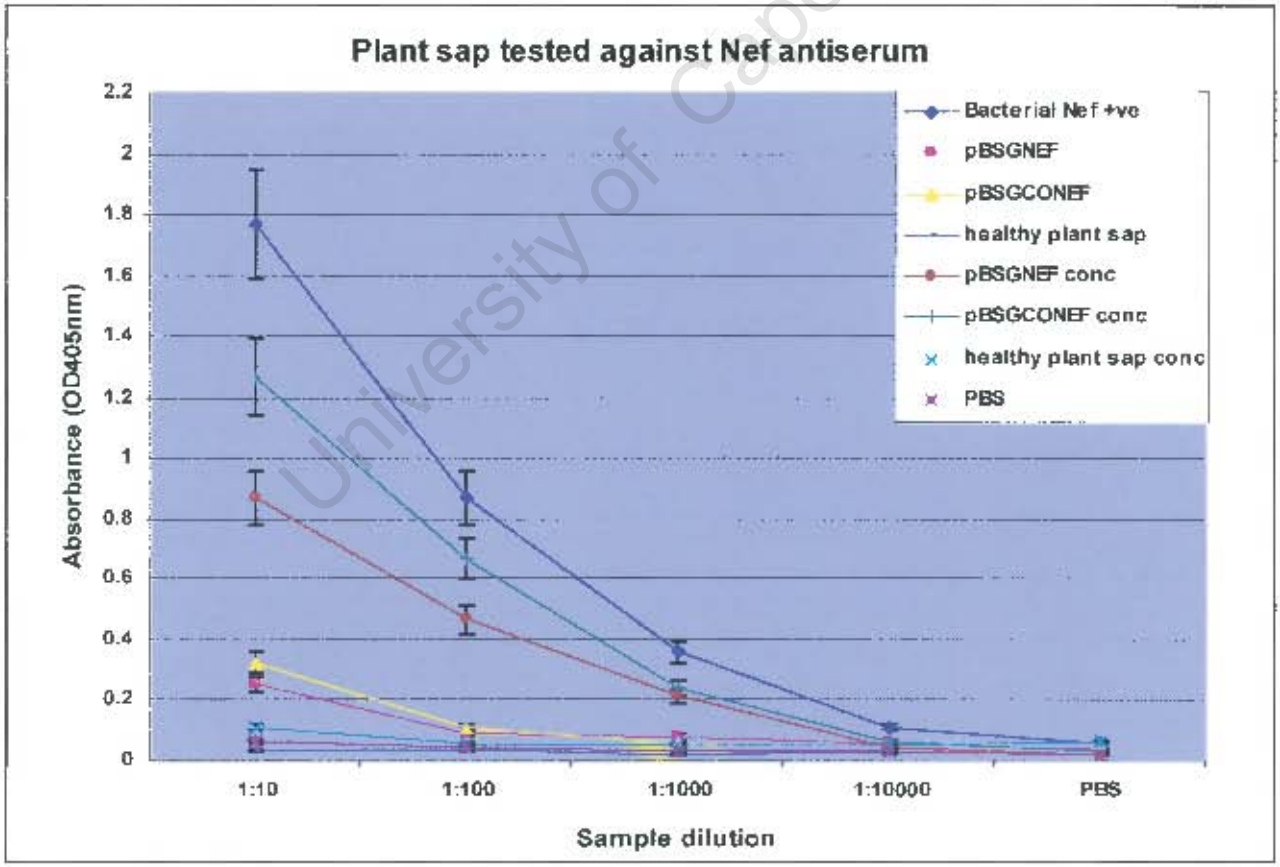
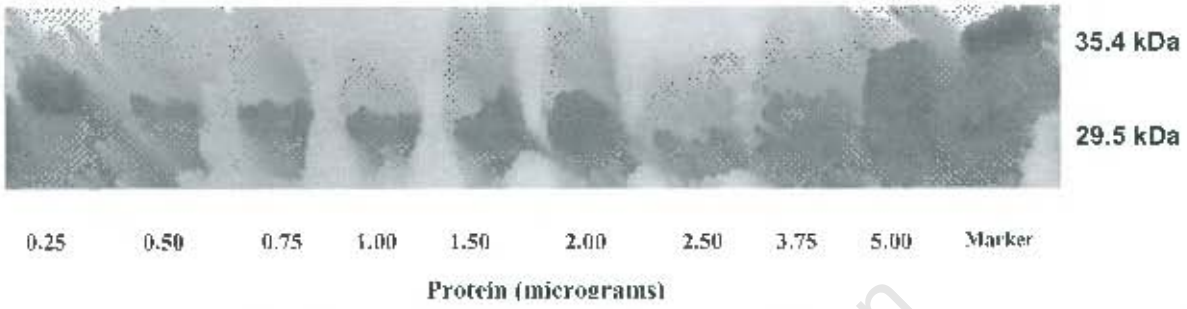


Figure 3.9: ELISA results of plant sap tested to Nef antiserum. The Nef +ve is bacterially expressed Nef. Filter concentrated plant samples are suffixed with the word conc.

3.3.8 Quantitation of Nef by western blot

Upper limit of DWR

A)



B)

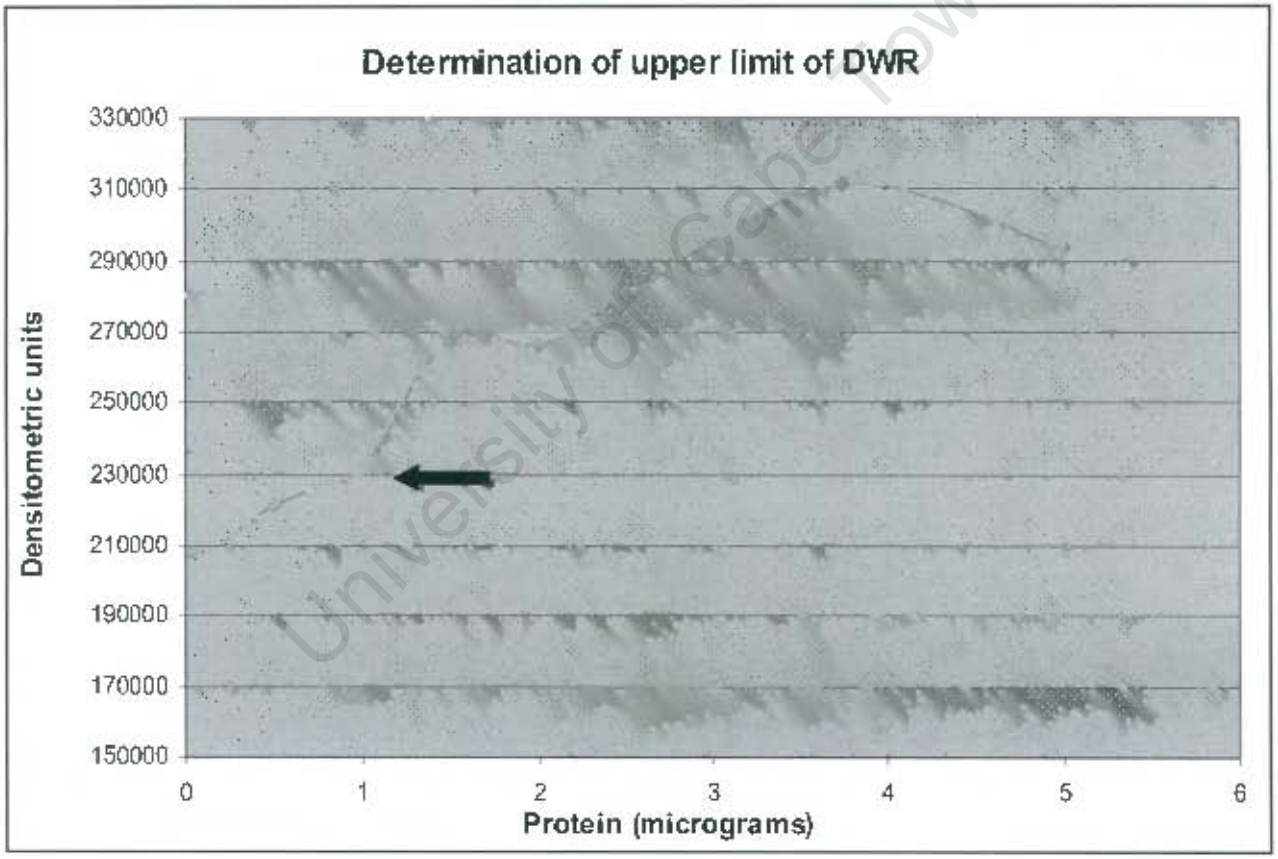
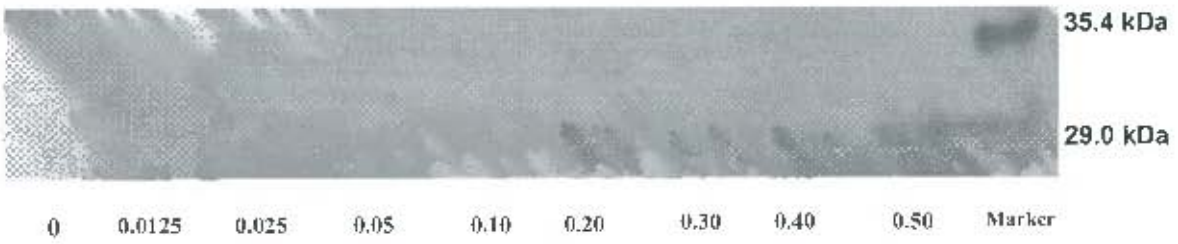


Figure 3.10: Determination of the upper limit of the DWR. The western blot of bacterially expressed Nef (A) was subjected to manual densitometric analysis and the results were plotted on a graph (B). The arrow shows the upper limit of the DWR.

Lower limit of DWR

A)



B)

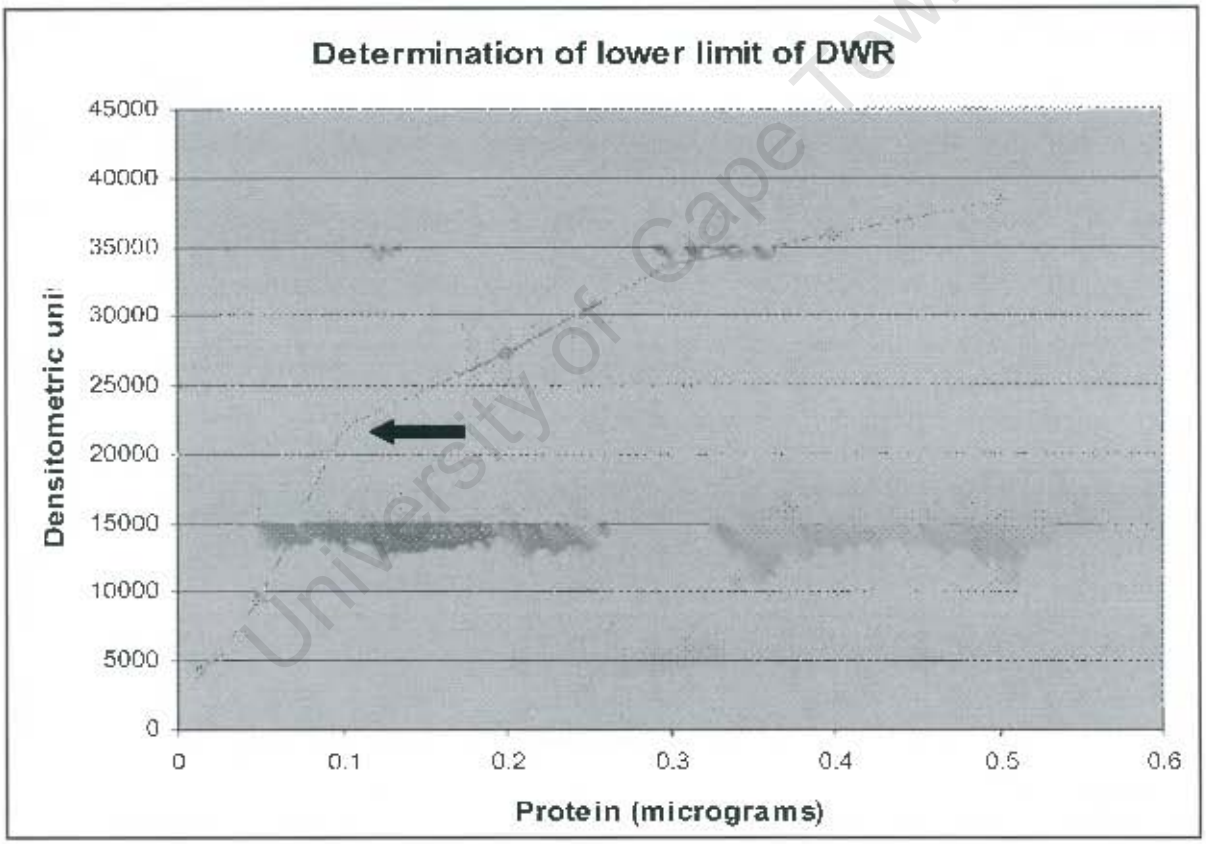
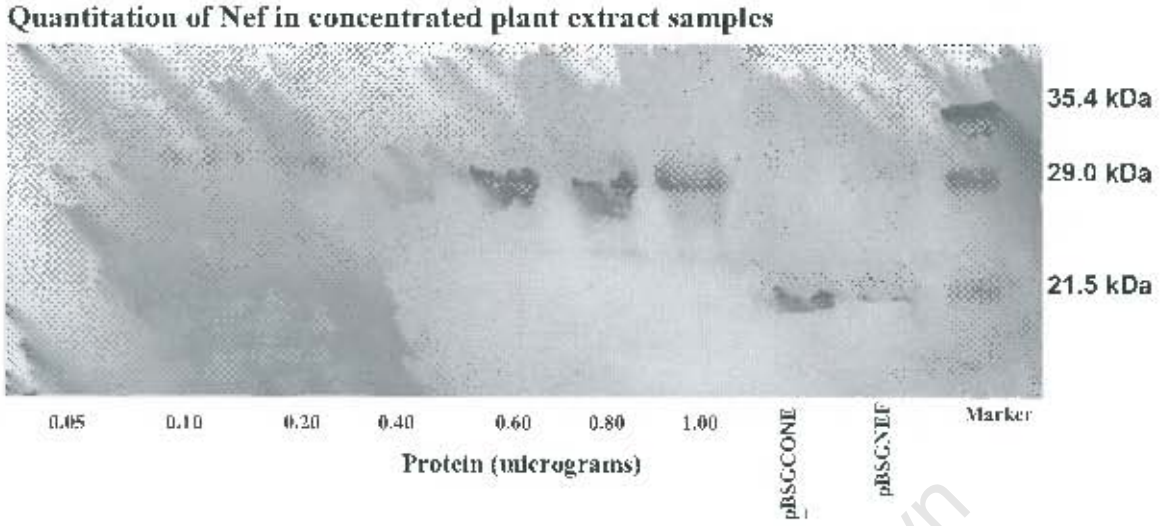


Figure 3.11: Determination of the lower limit of the DWR. The results from densitometric analysis of the western blot (A) are plotted on a graph (B). The arrow indicated the lower limit of the DWR.

A)



B)

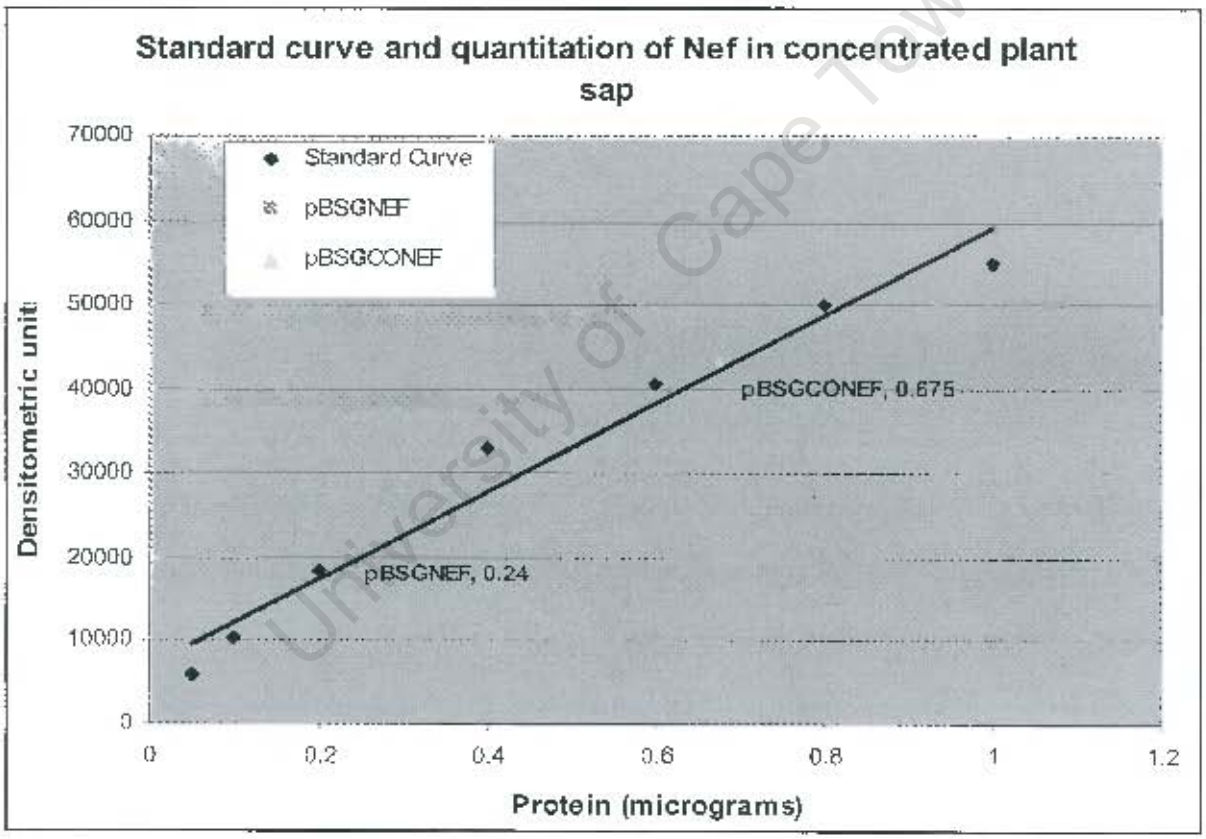


Figure 3.12: Quantitation of Nef in concentrated plant sap. The protein for the standard curve and the samples to be quantitated are loaded on the same western blot (A). The points of the standard curve were plotted on the graph with a computer generated smoothed curve (B). The densitometric values of the pBSGNEF and pBSGCONEF bands were used to estimate the total amount of the proteins loaded. Quantitation was repeated for another 2 infections. This figure shows the standard curve for infection A)

plant sap	Densitometric value from duplicate immunoblots	meanTotal Nef loaded (Interpolated on standard curve)	Mean total Nef loaded on western blot	Volume loaded on western blot	[Nef] in plant per gram of leaf (fresh weight)
pBSGNEF	A) 22945 B) 21641	0.24µg	0.238µg	30µl	0.159µg
	A) 22407 B) 27067	0.28µg			
	A) 23164 B) 20987	0.195µg			
pBSGCONEF	A) 39416 B) 40123	0.525µg	0.523µg	30µl	0.349µg
	A) 41770 B) 40049	0.495µg			
	A) 39229 B) 39711	0.55µg			

Table 3.2: Calculated mean levels of expression of Nef in plants expressing pBSGNEF and pBSGCONEF based on average quantitated levels in three separate infections of *Nicotiana benthamiana* with the two constructs.

Quantitation of levels of Nef expressed in *Nicotina benthamiana* was carried out by western blot after initially determining the DWR for the conditions under which the western blot was run.

In Figure 3.10 the upper limit of the DWR is established. The graph (Figure 3.10B) shows the densitometric values increasing in a linear fashion between 0.25 and 1.0µg to 230000 units after which there is a sharp jump in the densitometric reading of the band at 1.5µg to 270000 units. After this value there is no longer a linear relationship between the amount of protein added and the densitometric value generated by the band. The blot (Figure 3.10 A) itself becomes saturated and smudged such that densitometric values cannot be reliably read from 1.5 µg upwards. 1.0µg was designated the upper limit of the DWR.

The lower limit of the DWR was established in figure 3.11. There is a steady decline in the densitometric values of the bands from 0.5 to 0.1μg. There is a steep drop in the curve at values lower than 0.1μg (Figure 3.11B). The bands on the blot are barely visible below 0.1μg (Figure 3.11A) and therefore 0.1μg was designated the lower limit of the DWR.

Quantitation was then carried out by running standard curves on the same blot as the samples of interest. Figure 3.12 shows the quantitation of Nef produced in the first of three infections of *Nicotiana benthamiana* with pBSGNEF and pBSGCONEF. The standard curve was generated by running quantities of bacterially expressed Nef between 1.0 and 0.05 μg (figure 3.12A) along with the samples of concentrated plant extract of pBSGNEF and pBSGCONEF infected plants. The curve was plotted using an Excel spreadsheet and a smoothed curve was generated. Densitometric values generated from the Nef bands were used to generate an estimate of the total amount of Nef in the plant extract. The assay was carried out for three separate infections and the results were pooled and averaged in table 3.2. These figures were used to calculate the concentration of soluble and quantifiable Nef in each plant per gram of fresh tissue. The recombinant TMV vector pBSGNEF produced an average of 0.159μg of Nef, while pBSGCONEF produced 0.349μg of Nef per gram of fresh leaf tissue in *Nicotiana benthamiana*.

3.4 CONCLUSIONS

Climate and weather can be proclaimed the most important determinant factors for both plant production and crop productivity. However, in a greenhouse setting where these factors are controlled other factors become more significant. Because the desired product results from the product of a viral infection, the following characteristics of the plant varieties need to be considered.

Permissiveness: Infection depends on a lack of the host's ability to combat the infection. Because inoculation of plants may be the most expensive part of the process (due to the high price of *in vitro* transcription procedures), the ease and speed at which an infection is established could have a significant bearing on the price of the product.

Severity of symptoms: The severity of TMV disease symptoms has a bearing on the growth of the plant and therefore on the yield of protein to be harvested. In all plants the symptoms were pronounced but no significant difference in severity was observed between the two breeding lines. Furthermore, symptoms only became evident after the infection had become systemic and the plant was nearing readiness for harvesting.

Ontological and physiological changes: The basic aim of using this system was the generation of biomass containing rTMV or heterologous proteins encoded by the rTMV genome. For this purpose the plants used were chosen for infection during a period of high rates of vegetative growth which starts at 3 weeks after planting out of seedlings. Vegetative growth is a convenient overall term to describe all those activities associated with the generation and expansion of leaves, the formation of lateral apical meristems and the growth of some of these as branches and the concurrent expansion of the root system (Milthorpe and Moorby, 1979). Vegetative growth in any one axis is in almost all species terminated by flowering and it is clear that TMV accumulates poorly in flowering tissue (unpublished data). A longer sustained period of vegetative growth is therefore in the best interests of maximizing yields of biomass.

In the plants from LSB line, there were visible signs of the initiation of flowering after 14 days. In the UCT'95 line there were signs of flowering after 20 days post inoculation (figures 3.4 and 3.5). The change of an apex from vegetative to the flowering state

represents the outcome of an internal autonomous progression which is modulated to varying degrees, depending on the cultivar, by low temperature and photoperiod (Milthorpe and Moorby, 1979). In the presence of uniform temperatures and photoperiod the more rapid progression towards flowering of plants from the LSB batch can be attributed to a genetic predisposition to do so. As a result of the prolonged vegetative growth, the amount of biomass generated by the UCT'95 batch was on average 30% higher than that of the LSB batch.

In all aspects taken into consideration, the UCT '95 variety outperformed its LSB counterpart. While neither plant appeared TMV resistant, the UCT '95 variety seemed more permissive as the disease history appeared to be accelerated in this variety. Coupled with its longer period of vegetative growth, and resultant larger biomass, this translated into easier and quicker infections with recombinant TMV and greater yield of product when this variety was used. All these attributes made the UCT'95 cultivar a more flexible research tool and a better potential crop variety in the production of proteins using the TMV based vector system.

The HIV-1 *nef* gene constructed by Geneart™ to reflect an optimized codon usage for plants, and the wild type gene were successfully cloned into pBSG1057 as replacements of the *gfp* gene. RNA transcripts were made (figure 3.6) and these were used to inoculate *Nicotiana benthamiana* plants. Plants were harvested a few days after the control infections had shown systemic spread as the spread of the two constructs may have been slower than that of the pBSG1057.

RNA was extracted from plants infected with the two constructs and the control plants. The RNA was subjected to RT-PCR and the results confirmed the presence of *nef* mRNA in plants infected with pBSGNEF and pBSGCONEF (figure 3.7). This qualitative assay was unable to distinguish between intact rTMV genomes and sgRNAs encoding the insert gene.

Examination of the extract of plants infected with the two constructs by SDS-PAGE showed the presence of TMV coat protein. This was a sure sign of infection but no visible sign of the heterologous protein was present (Figure 3.8A lanes 5 and 6). Filtration of the plant extract using a Centricon® YM-30 centrifugal filter device and concentration using a Centricon® YM-10 centrifugal filter device produced a new protein band just below the hugely expanded TMV CP band at 21kDa (Figure 3.8A lanes 2 and 3).

Western analysis of the plant extracts from plants infected with pBSGNEF and pBSGCONEF confirmed the presence of TMV CP (Figure 2.8B). Nef was not detectable in the unconcentrated extract. Concentrated plant extract from both pBSGNEF and pBSGCONEF infected plants was shown to contain Nef (Figure 3.8 C lanes 2 and 3).
ELISA performed with anti Nef antiserum revealed the presence of Nef in the unconcentrated extract at low levels (figure 3.9) and an increased reaction was seen in the concentrated plant extract.

Nef was produced in the system by both constructs and accumulated at levels detectable by ELISA but detectable by western blot only after concentration of plant extract. A similar level of expression was obtained by Kasarev *et al.* (2001) in the production of Tat as a free protein using a TMV vector based system in *Nicotiana benthamiana.*

Quantification of protein by western blot

Quantification of heterologous protein can be done by several means. While sandwich ELISA would probably be the most accurate, it is rarely used for the quantitation of heterologous proteins in plants and there are few publications describing its use (Bruyns *et al.*, 1998). Researchers appear to favour western blotting or dot blots for quantitative measurements. Western blotting has some advantages in this particular case, as the resolving of proteins prior to blotting allows the separation of the protein of interest from the large quantities of TMV-CP that are present in the concentrated extract (figure 3.8A) prior to the immunoassay. It is likely that such a disproportionately large quantity of TMV-CP would interfere with the efficient immobilization of Nef in either ELISA or dot

blot assays. Pre-absorption with anti TMV-CP antibodies is likely to have been insufficient to remove such overwhelming quantities of CP and may have caused problems with reproducibility. Western blotting is also a well established technique in the laboratory and it requires small amounts of antiserum. The quantitation process was assisted by computer aided manual densitometric analysis of bands generated by capturing developed membranes with Genegenius® bio imaging system and subjecting them to readings using the GeneTools version 3.00.13 program from Syngene®.

The dynamic working range for quantitation using western blotting was determined to lie between 0.1 and 1.0µg of protein using the bacterially expressed His(6)-Nef as a control reagent. Standard curves were set up using this reagent and these were run on each gel with the samples being quantitated. There is a difference in the electrophoretic mobility of the proteins being quantitated and those of the standard curve. The reduced mobility of the bacterially expressed His(6)-Nef is largely due to the hexa-histidine tag and differences in posttranslational modifications the protein may undergo in the two systems such as glycosylation, sulfation and phosphorylation. The actual difference in molecular weight between the two is 3.8kDa. This difference is negligible in the context of quantitation of total protein from the standard curve. Samples from three different infections were tested and averages of 159ng for pBSGNEF and 349ng of Nef per gram of fresh leaf tissue in *Nicotiana benthamiana* with pBSGCONEF were the calculated yields. The level of expression amounts to less than 0.0007% of total soluble protein. This is low when compared with expression of the therapeutic antibody 38C13 (ScFv) by McCormick *et al.* (1999) in tobacco using a TMV based system. 38C13 (ScFv) is a B-cell lymphoma treatment and idiotype vaccine and it was produced using a TMV subgenomic coat protein promoter and a Rice a-amylase signal sequence at 30.0 µg/g leaves *Nicotiana benthamiana*. Nemichov *et al.* (2000) showed that plants infected with recombinant tobacco mosaic virus (TMV) engineered to express the HVR1/CTB chimeric protein, contained intact TMV particles and produced the HVR1 consensus peptide fused to the functionally active, pentameric B subunit of cholera toxin. In these experiments, the amounts of chimeric CTB per 100 mg of total soluble protein varied within a range of 70–950 ng, which is 0.07–0.9% of TSP and approximately 6 to 80mg

per gram of leaf tissue. A 10-fold increase in protein concentration in the plant extract was also obtained using Centriprep concentrators (YM-10). Using the same vector, Jaffray *et al.* (2003) was able to produce HIV-1 subtype C Gag VLPs similar to those produced in baculovirus. These were found to accumulate at a rather low 5ng/g of leaf tissue, which was insufficient to succesfully isolate and conduct immunogenicity experiments. The low yield may be attributable to the relatively large size of the *gag* gene relative to *nef* and genes used to express plantibodies for example. There are no reports in the literature of expression of free protein using the TMV vector system to levels exceeding 80µg/g fresh weight of leaves. However, encouraging results have been obtained by fusing heterologous genes to the coat protein of TMV which is known to accumulate to high levels in infected leaves. Using this approach, researchers have reported expression levels as high as 800µg/g of fresh weight (Pogue *et al.*, 1998). The subsequent chapter will explore this approach further.

Codon optimised vs wild type *nef*

The DNA sequences of the wild type and codon optimized sequences have about 75.82% identity. The codon optimized gene contains 110 silent nucleotide changes compared to its DU151 counterpart used in this study. Results in this work show an improvement in expression with the use of the plant optimized gene. However, codon optimization is in its infancy and with current knowledge, it is difficult to determine at which level codon usage is most influential. Stability of the DNA helix, mRNA stability, amino acid composition and codon choices are all factors which will affect or be influenced by codon usage (Boudraa and Perrin 1987). While DNA stability may be influential in transgenics, it is not a factor in transient systems such as the TMV-based vector system. However, because the heterologous gene in the vector is translated off a subgenomic RNA, it is possible that the stability of this species is enhanced by the optimized sequence. The translatability of the mature mRNA has implications on its accumulation in transgenic plants. Unfortunately, current knowledge of parameters affecting RNA stability is restricted in plants (Vancanneyt *et al.*, 1990). The relationship between the host amino acid composition and its codon usage is difficult to gauge (Boudraa and Perrin 1987) and so little can be said in relation to this.

In *E. coli*, yeast and plants, synonymous codon choice patterns are related to the abundance of isoaccepting tRNAs and this is positively correlated to the level of gene expression (De Amicis 2000). This last factor may possibly be the most significant in improvement of expression in the transient expression system.

Enhancement of expression achieved by optimization of the codon usage was such that expression levels still remained at levels well below the level of 1% of soluble protein that is needed for commercial feasibility (Henry Daniell, 2001). Future investigations may explore the option of optimizing the codon usage to that of TMV to increase expression further. Virus genomes frequently have a G+C content significantly different from that of their host species, reflecting a different codon usage pattern of unknown significance (Strauss *et al.*, 1995). DNA shuffling technology and mutagenesis have both been used in improvement of the movement protein of TMV (Toth 2002). This has resulted in the development of a movement protein vastly improved in its stability and efficiency. While the same technology could be applied to heterologous genes for improved expression it is also likely that the improved vectors that will result from increased efficiency of the movement and other proteins will enable greater expression.

CHAPTER 4

Expression of Nef CTL epitopes as TMV coat protein fusions

4.1 INTRODUCTION

4.1.1 TMV Coat Protein

The abundance of TMV CP in infected plants and the purification advantages presented by the unique physical properties of the TMV virion structure make it an ideal candidate for a vaccine epitope display system. The structure of TMV has been refined at 2.9A from X-ray fiber diffraction studies. A right handed helix is formed from about 2130 identical coat protein (CP) subunit of 17.5kDa (Cunningham and Porter, 1998). The TMV rod structure is illustrated in figure 4.1A.

The core of a single TMVCP subunit (figure 4.1B) contains a bundle of four antiparallel α helices projecting away from the RNA binding site. The region at high radius contains the N and C termini as well as a loop connecting the so-called right-slewed (RS) and right-radial (RR) α-helices. This four helix domain is one of the more common and simple protein folds. The stability of the structure is based on the packing of hydrophobic side chains on the interior and the orientation of hydrophilic groups on the surface (Cunningham and Porter, 1998).

The structure of the CP subunit and the rod shaped viruses offer flexibility for the location and context that antigenic peptides are presented by fusion options on the N and C terminal regions and with constrained surface exposed loop regions. The density display is unparalleled in these systems with greater than 2100 peptides exposed per virion. In fact, this high density is potentially problematic with the solubility of the particles being affected. Where such issues are relevant, an alternative strategy has been employed to counter this effect. A "leaky read through" amber codon at the end of the TMV coat protein gene will promote the translational extension of a genetic fusion at a ratio of every $1/10^{th}$ to $1/20^{th}$ coat protein (Turpen *et al.*, 1995) as opposed to a direct C-terminal fusion where all coat proteins possess the peptide fusion. The less dense display of peptide additions from these C-terminal approaches may promote better virion solubility and/or assembly (Turpen *et al.*, 1995). As illustrated in the literature review, results from immunogenicity assays on antigens generated using the epitope display

method have already shown much promise. The Vaccine Group at the University of Cape Town is also interested in developing as part of the vaccine effort, immunogens that are able to induce production of high levels of HIV specific antibodies for neutralization as well as CTL responses using this method.

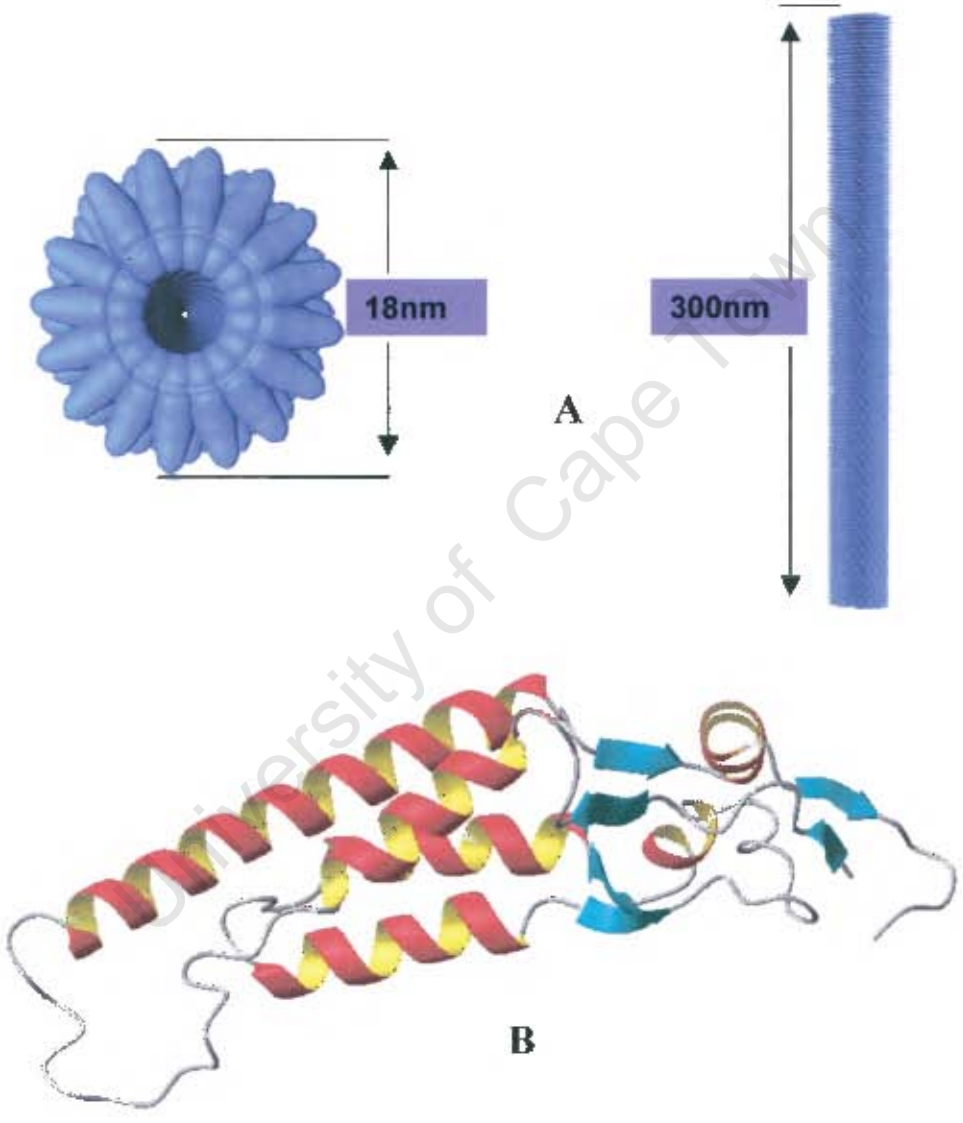


Figure 4.1: Cartoons representing the structure of TMV virus particles (A) and the TMV CP subunit tertiary protein structure (B). The region at high radius on the TMV CP subunit is on the right hand side (Taken from www.virology.net)

To this end, intermediate cloning vectors for cloning additional oligonucleotides or DNA sequences to the 5' or 3' ends of the TMV CP gene in pBSG1057 were constructed (F. Tanzer, University of Cape Town). This allowed for the attachment of epitopes on the N and C termini of the TMV coat protein encoded by pBSG1057. In this section, I aimed to investigate the possibility of expressing two HIV-1 Nef CTL epitopes using the plant viral epitope display method.

4.1.2 Selection of Nef CTL epitopes

The Nef protein is very immunogenic and is rich in CTL-inducing epitopes. Several studies relating to the mapping of the immunogenic regions of the protein are reviewed in Chapter 1. For this purpose the most immunodominant and frequently recognised peptide in a study by Mashishi *et al* (2001) was chosen as the primary epitope. The peptide YKAAFDLSHFLKEKG constitutes the H1 helical domain of the Nef core (Grzesiek *et al.*, 1997) (also see figure 1.3) and was recognised by 31% of individuals tested. It was designated NP31.

A second epitope EEPEVGFPV was designated EP1. This peptide is a part of the epitopic region that was recognised by 15% of tested individuals and forms part of the type II polyproline helix domain that is the first major domain after the protease cleavage site in Nef. This second peptide is smaller and should have a slightly less constrained structure than NP31.

The aim was therefore to construct and purify four rTMVs from pBSG1057 with each bearing CP subunits decorated with one of the two peptides as N or C terminal fusions. Such rTMVs would present CTL epitopes from Nef at a high density on a large particulate antigen. This would make an ideal ingredient for a candidate vaccine that seeks to induce some form of cellular immunity against HIV infection. Should the particles be successfully purified, a further aim was to test their immunogenicity in small animals such as mice.

4.2 MATERIALS AND METHODS

4.2.1 Construction and cloning of Nef epitope gene fusions

The process by which the Nef epitope gene fusion vectors were created is illustrated in figure 4.2, which also shows the construction of pBSGN5NP31 and pBSGC3NP31. The same process was followed in the construction of pBSGN5EP1 and pBSGC3EP1.

A. Preparation of vectors for creation of coat protein fusion constructs

Intermediate vectors used in the construction of coat protein fusion genes were constructed by Dr Fiona Tanzer in the Department of Molecular and Cell Biology at UCT as follows: The coat protein gene in the pBSG1057 vector was excised using the *Sal* 1 and *Pst* 1 restriction sites. The excised region was subcloned into a standard pUC19 cloning vector. The TMV coat protein gene in the pUC19 vector was then modified by site directed mutagenesis via PCR such that a cloning site with the restriction sites *Nru* 1 and *Not* 1 immediately adjacent to each other with the *Nru* 1 site on the 5' side were introduced. This cloning site was separately introduced at both the 5' and the 3' end of the coat protein gene and designated pUCN5 and pUCC3 respectively (figure 4.2A). These vectors would allow the introduction of a gene in frame with the coat protein gene to be expressed as either a C or N terminal fusion.

B. Construction of *nef* epitope oligonucleotides

The sequences of the oligopeptides selected for expression as coat protein fusions (NP31:YKAAFDLSHFLKEKG and EP1:EEPEVGFPV) were entered into the DNAman® (LynonBiosoft) version 4.13 computer program and the expected DNA sequences reflecting a plant codon bias were obtained using this program. Where there were ambiguities, individual nucleotides were selected based on a codon usage table for *Nicotiana benthamiana* found on the NCBI web-page. In each case the codon with the highest usage or fraction was selected so as to truly reflect the *Nicotiana benthamiana* codon usage and bias. The resulting sequence was then modified to include on the 5' end a sticky end of a cut *Nru* 1 site and on the 3' end, a sticky end of a cut *Not* 1 site.

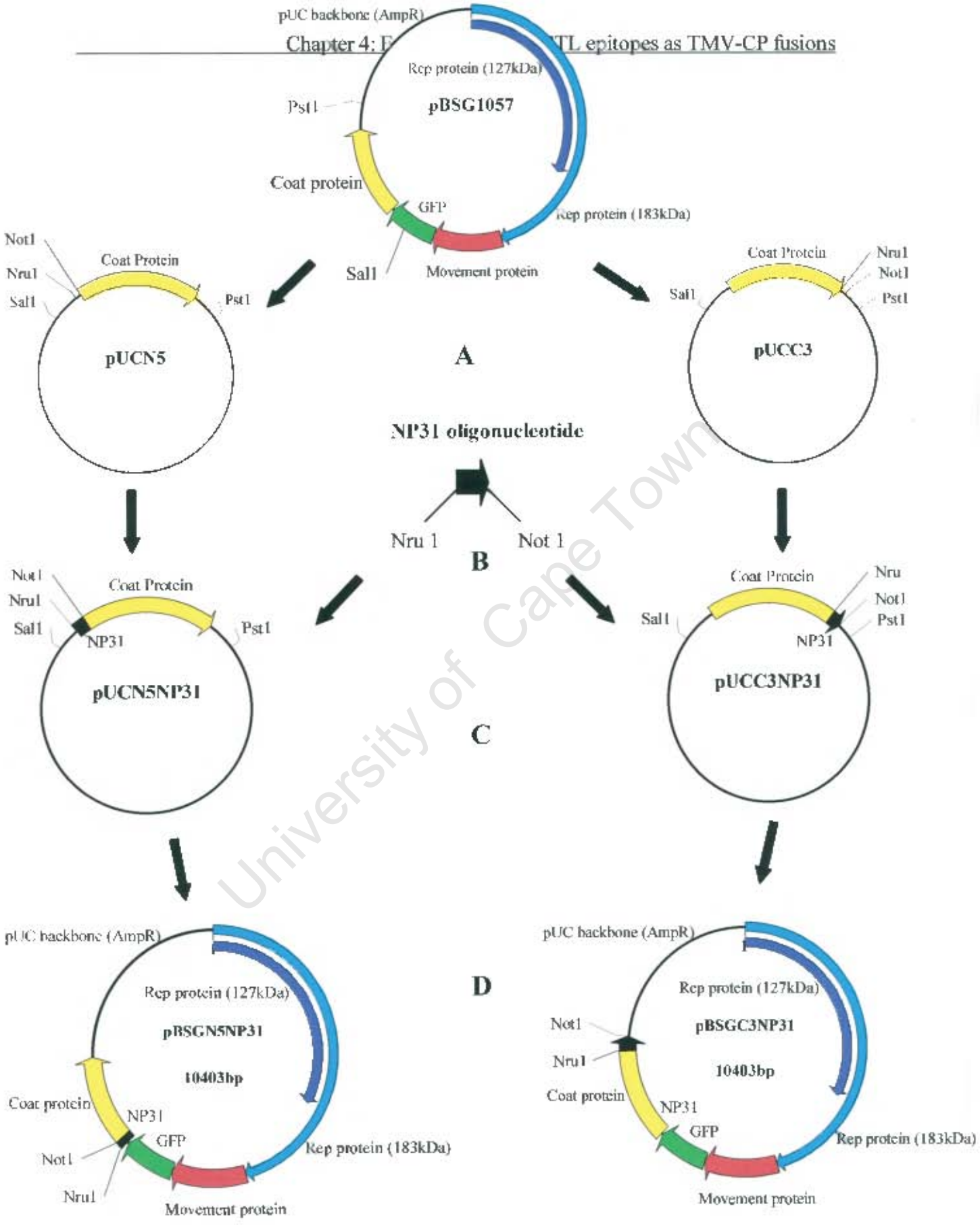


Figure 4.2: Construction of CP epitope fusions pBSGN5NP31 and pBSGC3NP31

The following oligonucleotides were synthesized in the Oligonucleotide Synthesis Laboratory of the Molecular and Cell Biology Department, UCT.

NP31f 5'-TACAAGGCTGCTTTTGATCTCATTTTCTGAAGGAAAAG-3'

NP31r 5'-CTTTTCCTTCAGAAAATGAGAATCAAAAGCAGCCTTGTA-3'

EP1f 5'-CGAGAAGAACCAGAAGTTGGTTTTCCAGTTGC-3'

EP1r 5'-GGCCGCAACTGGAAAACCAACTTCTGGTTCTTCTCG-3'

The two NP31 and the two EP1 oligonucleotides were both annealed by heating to 95°C for 10 minutes and snap cooling on ice.

C. Ligation of oligonucleotides with the fusion vectors.

The annealed oligonucleotides were then cloned into pUCN5 and pUCC3 as follows. 1µg of pUCN5 and 1µg of pUCC3 DNA were digested with 1 unit each of *Not* 1 and *Nru* 1. The digested DNA was gel purified in a 0.8% TBE agarose gel (appendix A). The annealed oligonucleotides were ligated to the digested pUCN5 and pUCC3 DNA by incubating the DNA at a vector to insert ratio of 1:8. This gave excess of oligonucleotide in relation to the vector and reduced the amount of self ligation by any vector molecules not fully digested with both enzymes. The resulting ligated products were used to transform high efficiency DH5α competent cells and plated onto ampicillin plates. Resulting colonies were picked and grown overnight and plasmid DNA was extracted by the miniprep method.

Screening of the plasmid DNA was by PCR using the following primer sets.

pUCN5NP31

Forward primer: 5'-TACAAGGCTGCTTTTGATCTG-3'

Reverse primer: 5'-CTATTGTTGTGAGATTTCC-3'

pUCC3NP31:

Forward primer: 5'-CCTTATACAATCAACTCTCCGAGCC-3'

Reverse primer: 5'-CTTTTCCTTCAGAAAATGAGACAG-3'

pUCN5EP1

Forward primer: 5'-CGAGAAGAACCAGAAGTTGGTTTTCCAGTTGC-3'

Reverse primer: 5'-CTATTGTTGTGAGATTTCC-3'

pUCC3EP1

Forward primer: 5'-CCTTATACAATCAACTCTCCGAGCC-3'

Reverse primer: 5'-GGCCGCAACTGGAAAACCAACTTCTGGTTCTTCTCG-3'

Clones showing a positive PCR reaction were sequenced to confirm the presence of the coat protein fusion gene in frame. Confirmed clones were designated pUCC3NP3, pUCN5NP3, pUCC3EP1 and pUCN5EP1.

D. Insertion of coat protein fusions into pBSG1057

1µg each of pUCC3NP3, pUCN5NP3, pUCC3EP1 and pUCN5EP1 DNA was digested with *Sal* 1 and *Pst* 1. This excised the TMV DNA incorporating the CP fusions from these constructs and the DNA was gel purified from a 0.8% TBE agarose gel. The ends of the purified DNA fragment were dephosphorylated by incubating the DNA with 1 unit of shrimp alkaline phosphatase (SAP) (Roche) for 30 minutes at 37 C. The phosphatase was inactivated by incubating the solution at 65 C for 15 minutes. 1µg of pBSG1057 DNA was also digested with the same enzymes and the plasmid backbone without the CP gene was similarly gel purified. The dephosphorylated *Sal* 1/*Pst* 1 coat protein gene fragments were ligated to the *Sal* 1/*Pst* 1 pBSG1057. The ligated DNA was used to transform high efficiency DH5α competent cells and plated onto ampicillin plates. Resulting colonies were then picked and grown overnight and the plasmid DNA extracted by the miniprep method. The presence of terminal fusions on the coat protein gene was checked using the same primers used in the preceding section C. Clones with DNA yielding positive PCR reactions were designated pBSGN5NP31, pBSGC3NP31, pBSGN5EP1 and pBSGC3EP1 respectively. All the pBSG1057-based fusion constructs retained the *gfp* gene which acted as a marker of infection.

4.2.2 *In vitro* transcription of constructs

Large-scale plasmid DNA extractions of pBSG1057, pBSGN5NP31, pBSGC3NP31, pBSGN5EP1 and pBSGC3EP1 respectively were carried out by the Nucleobond™ method. Production of RNA transcripts from pBSG1057, pBSGN5NP31, pBSGC3NP31, pBSGN5EP1 and pBSGC3EP1 and a luciferase gene under the control of a T7 RNA polymerase promoter as a control DNA was carried out using the RIBOMAX™ Large Scale RNA Production Kit (Promega) by the method specified by the manufacturer (Appendix C). The success of the *in vitro* transcription was determined by electrophoresis of transcription products on a 0.8 % TBE agarose gel.

4.2.3 Mechanical infection of *Nicotiana benthamiana*

Three week old *Nicotiana benthamiana* plants at the four leaf stage from the UCT'95 line were used as host plants. The plants were mechanically inoculated by micro-abrasion using 50µl of the RNA reactions and 50µl of DEPC water per plant. Inoculation was carried out using autoclaved cotton buds. The cotton buds were dipped in baked celite and then into the RNA solution and gently rubbed onto the leaves in such a way that there was no visible abrasion of the leaf tissue. Inoculations were carried out with RNA transcripts of pBSG1057 as a positive control, pBSGN5NP31, pBSGC3NP31, pBSGN5EP1 and pBSGC3EP1 and with water as a negative control. The plants were maintained in the UCT Molecular Biology plant rooms. All constructs contained the *gfp* gene and therefore all plants were monitored daily for the presence of symptoms and the appearance of GFP. Visualisation of GFP was done in a dark room with a long wavelength ultraviolet lamp. At 14 days after inoculation, leaves were harvested and tested for the presence of recombinant CP RNA and protein.

As a bioassay for the production of virus particles *in vivo*, the infectivity of homogenates of inoculated leaves showing fluorescence was tested on fresh young plants. Plants were monitored for symptoms of infection and fluorescence for 2 weeks.

4.2.4 RNA Extraction, reverse transcription and PCR amplification of cDNA

Innoculated leaves showing expression of GFP were harvested for RNA extraction and detection of proteins. 100mg of leaf material from a harvested plant was placed in a mortar and homogenized in liquid N_2. RNA was extracted using the TRIZOL method (appendix A).

RNA extracted from each plant was reverse transcribed and the resulting cDNA amplified by PCR using C. therm™ DNA polymerase™ (Roche™). These reactions were carried out in a single tube for each RNA sample in a thermocycler a one step RT-PCR procedure formulated by Roche ™ (appendix A). As a positive control for the RT-PCR reaction, RNA extracted from the pBSG1057 infected plants was subjected to RT-PCR using an internal *gfp* primer set.

For the remainder of the constructs, the following primer sets were used.

pBSGN5NP31

Forward primer: 5'-TACAAGGCTGCTTTTGATCTG-3'

Reverse primer: 5'-CTATTGTTGTGAGATTTCC-3'

pBSGC3NP31:

Forward primer: 5'-CCTTATACAATCAACTCTCCGAGCC-3'

Reverse primer: 5'-CTTTTCCTTCAGAAAATGAGACAG-3'

pBSGN5EP1

Forward primer: 5'-CGAGAAGAACCAGAAGTTGGTTTTCCAGTTGC-3'

Reverse primer: 5'-CTATTGTTGTGAGATTTCC-3'

pBSGC3EP1

Forward primer: 5'-CCTTATACAATCAACTCTCCGAGCC-3'

Reverse primer: 5'-GGCCGCAACTGGAAAACCAACTTCTGGTTCTTCTCG-3'

pBSG1057

Forward primer: 5'-GGTTTAATTAAAATGGAGCCAGTAGATCCTAG-3'

Reverse primer: 5'-GGATCTCGAGGATATCCTCCACCTTCTTCTTCG-3'

4.2.5 Harvesting of leaves for detection of proteins

100mg of leaves were crushed and homogenized in liquid nitrogen by mortar and pestle. This homogenized leaf material was suspended in 0.5ml of PBS and vortexed. The homogenate was centrifuged in a microfuge for a minute at room temperature to remove large particulate matter and the supernatant plant sap recovered. The plant sap was placed in SDS-PAGE loading buffer (Appendix A12) and boiled for 10 minutes. 10µl of boiled plant extract in loading buffer was loaded per well of a 15% SDS-PAGE gel and electrophoresed.

4.2.6 Western Blotting

Proteins resolved by SDS-PAGE were transferred onto nitrocellulose by the semi-dry blotting method and tested to antisera by the western blotting method in appendix F. The polyclonal antisera tested against the plant sap are listed below in table 4.1. All SDS-PAGE gels were run in duplicate with one of the gels being blotted while the other was stained with coomassie blue for visualisation of the proteins. All antisera were diluted in blocking buffer with 2% milk powder.

Primary antibody	Raised in	Source	Dilution	Secondary antibody	Source	Dilution
Anti TMV	Rabbit	MCB Dept UCT	1:1000	Goat anti rabbit	Sigma	1:5000
Anti *gfp*	Mouse	Qiegen	1:5000	Goat anti mouse	Sigma	1:10000

Table 4.1: Polyclonal antisera used in western blots on plant extract

4.3 RESULTS AND DISCUSSION

4.3.1 Construction and cloning of Nef epitope gene fusions

The intermediate vectors pUCC3 and pUCN5 were digested with *Nru I* and *Not I*. The results of digestion with *Nru I* and *Not I* are shown in figure 4.3. Because of the proximity of the restriction sites, digestion at both sites appears similar to a single enzyme digestion. The digestion was therefore carried out for a prolonged time to ensure digestion with both enzymes. Since the synthetic oligonucleotides used in the ligations were unphosphorylated, the digested vector was not treated with alkaline phosphatase to prevent self ligation. Rather, the excess of annealed synthetic oligonucleotide ensures the prevention of vector relegation in the event that any vector molecules were digested with only one of the two enzymes. This results in a large number of positive colonies from the subsequent transformation of DH5α with the ligation mix.

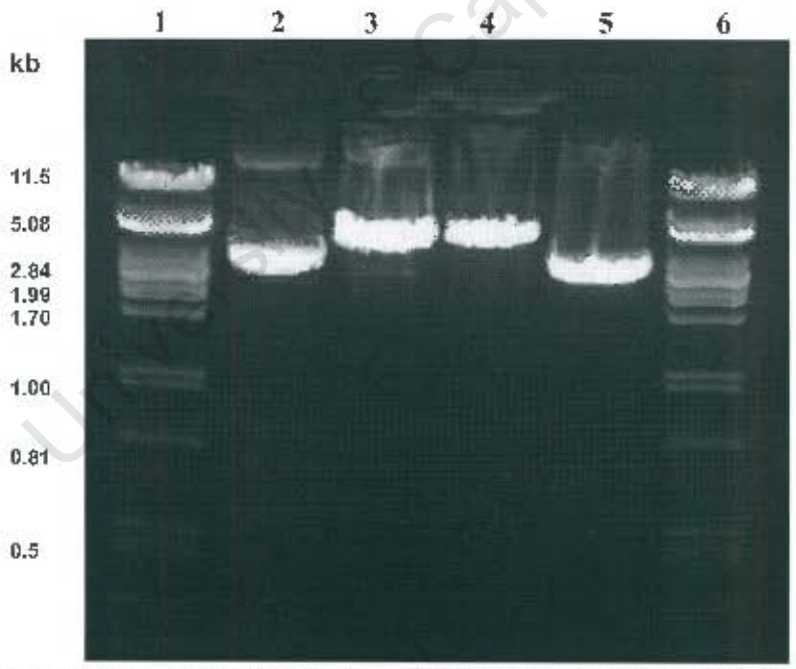


Figure 4.3: pUCC3 and pUCN5 digested with *Nru* 1 and *Not* 1. Lane 1, λ/*Pst* 1 DNA marker; Lane 2, undigested pUCC3; Lane 3, pUCC3 digested *Nru* 1/ *Not* 1; Lane 4, pUCN5 digested *Nru* 1/ *Not* 1; Lane 5, undigested pUCN5 and lane 6, λ/ *Pst* 1 DNA marker.

Screening of the colonies by PCR resulted in a high proportion of positive reactions. Figure 4.4 shows an agarose gel with results the PCR screening of clones resulting from

ligation of pUCC3 with the oligonucleotide NP31. One sample of extracted DNA yielding positive results was sequenced to confirm the presence of the oligonucleotide and to establish that it was inserted in frame. The sequenced DNA was used in the construction of the pBSG1057 based vector.

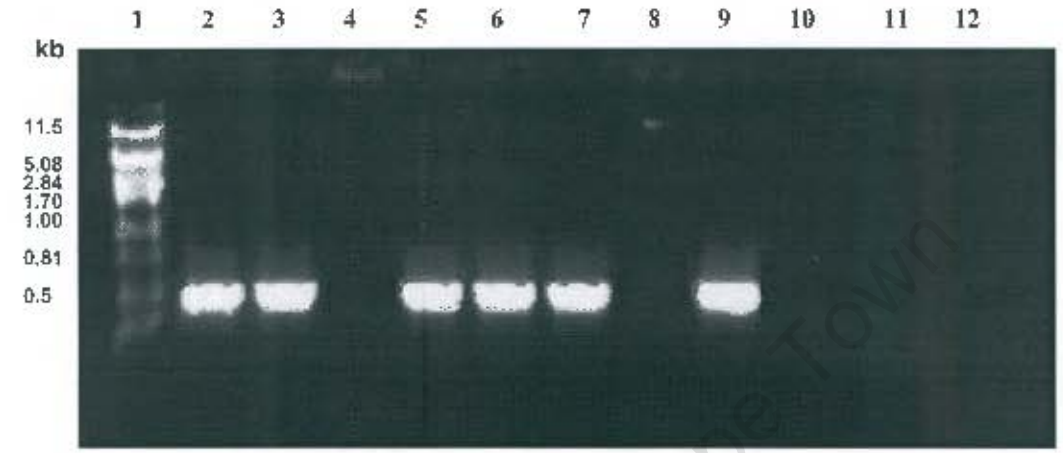


Figure 4.4: Results of PCR screening of clones resulting from ligation of pUCC3 with NP31. Lane 1, λ/*Pst* 1 DNA marker; Lanes 2-8, PCR product from 8 colonies resulting from pUCC3/NP31 ligation reaction; Lane 10, DNA from 8 screened colonies subjected to PCR with no forward primer; Lane 11, no reverse primer and lane 12, no Taq polymerase.

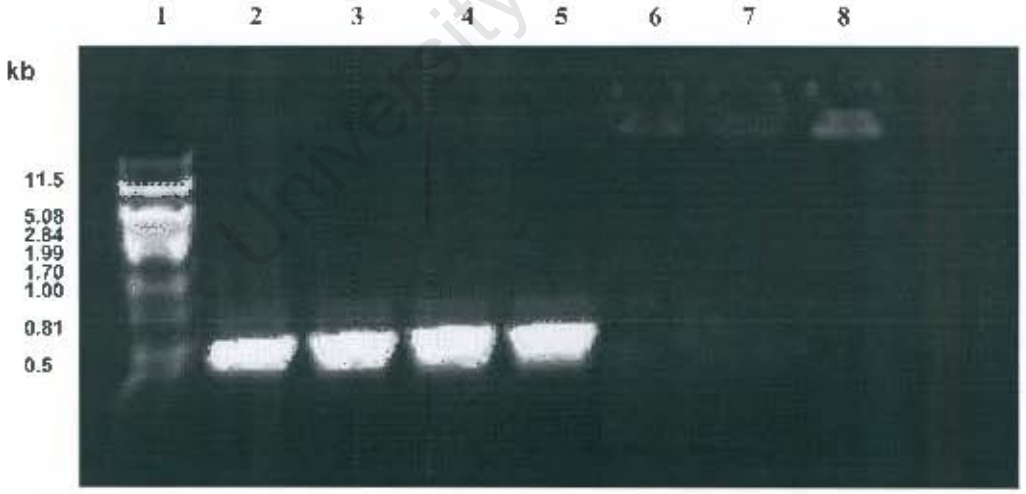


Figure 4.5: Agarose showing a final PCR check of pBSGN5NP31, pBSGC3NP31, pBSGN5EP1 and pBSGC3EP1. Lane 1, λ/ *Pst* 1 DNA marker; Lane 2 pBSGN5NP31; Lane 3, pBSGC3NP31; Lane 4, pBSGN5EP1; Lane 5, pBSGC3EP1; Lane 6, all template DNA's, reverse primers and no forward primers; Lane 7, all template DNA's forward primers and no reverse primers and Lane 8, No DNA.

Sequenced DNA from intermediate vectors pUCC3NP31, pUCN5NP31, pUCC3EP1 and pUCN5EP1 was used to construct the pBSG1057 based vectors. Figure 4.5 shows the digestion of these vectors and pBSG1057 with *Sal* 1 and *Pst* 1.

Ligation of the *Sal* 1/*Pst* 1 insert from the intermediate vectors and Sal 1/Pst 1 digested pBSG1057 backbone resulted in formation of the vector constructs pBSGC3NP31, pBSGN5NP31, pBSGC3EP1 and pBSGN5EP1. Extracted DNA from colonies harbouring these constructs was tested by PCR to ensure the presence of the oligonucleotides in the vectors before embarking on large scale preparations of DNA for transcription of RNA. Figure 4.6 shows the results of the screening of the four constructs.

4.3.2 *In vitro* transcription of constructs

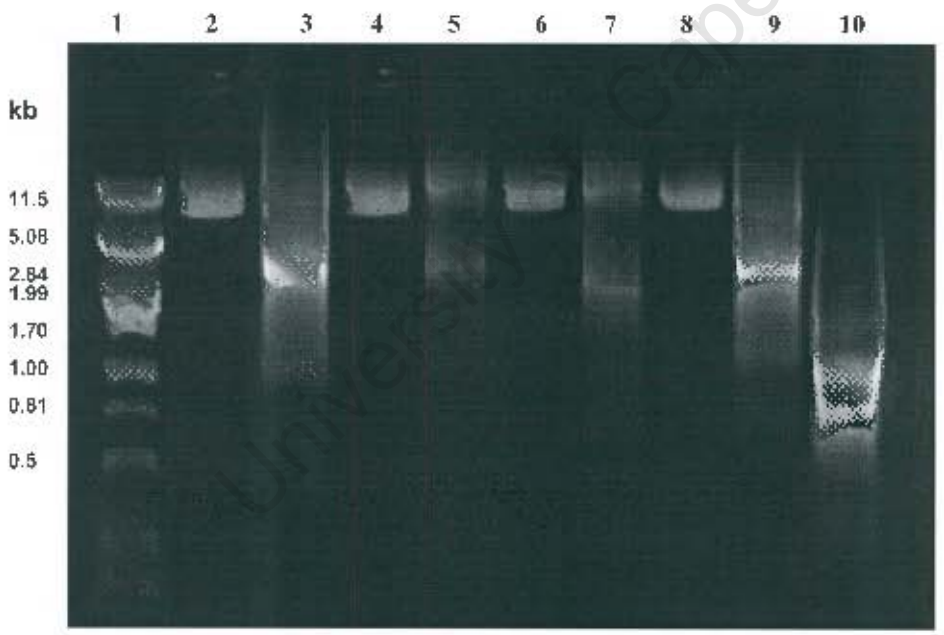


Figure 4.6: Results of in-vitro transcription of pBSGN5NP31, pBSGC3NP31, pBSGN5EP1 and pBSGC3EP1. Lane 1, λ/ Pst 1 DNA marker; Lane 2, pBSGN5NP31 DNA; Lane 3, pBSGN5NP31 RNA; Lane 4, pBSGC3NP31 DNA; Lane 5 pBSGC3NP31 RNA; Lane 6, pBSGN5EP1 DNA; Lane 7 pBSGN5EP1 RNA; Lane 8, pBSGC3EP1 DNA; Lane 9, pBSGC3EP1 RNA and Lane 10, manufacturers control plasmid transcription.

In vitro transcription of large scale preparations of the constructs pBSGC3NP31, pBSGN5NP31, pBSGC3EP1 and pBSGN5EP1 resulted in generation of sufficient RNA transcript to initiate an infection by manual inoculation of plants. Figure 4.7 shows the results of the in vitro transcription on a 0.8% TBE agarose gel. 1µl of the RNA transcript is loaded onto the gel and so while the levels of RNA in lanes 5 and 7 may be low in relation to lanes 3 and 9, it is more than sufficient to cause infection in young plants.

4.3.3 Mechanical infection of *nicotiana benthamiana*

The inoculation of plants with RNA from the two fusion constructs pBSGN5NP31 and pBSGC3NP31 resulted in attenuated infections in comparison to the pBSG1057 control infection. The initial appearance of symptoms by way of fluorescent spots was delayed in both fusion constructs, with the first fluorescent spots appearing after 7 days. The spots grew at a slower pace than control (pBSG1057 infected) plants and the cell to cell spread of the infection was insufficient to cover the entire inoculated leaf (figure 4.7).

The infection from the fusion constructs remained confined to the inoculated leaf and appeared to only spread along the vein of the leaves and progress towards the stem starting at about 10 days post inoculation. The fluorescence never went beyond the inoculated leaf even when left to progress for longer than 3 weeks. There were no major differences between the N-terminal and C-terminal fusion constructs.

Repeated attempts to passage the virus and bulk up the infection using homogenates of infected leaves from both fusion constructs failed to produce a new infection.

Inoculation of plants with transcripts of pBSGN5EP1 and pBSGC3EP1 produced results similar to those seen in the previous infections. There were few notable differences, namely, infections appeared to manifest quicker with fluorescent spots appearing after 5 days and the fluorescence appeared to cover a slightly larger area of the leaf. Although expression of GFP was mainly concentrated near the veins and towards the stem, there was a more pronounced outward spread of GFP into the leaf from infection loci. However, several fundamental similarities persisted. The infections were much slower

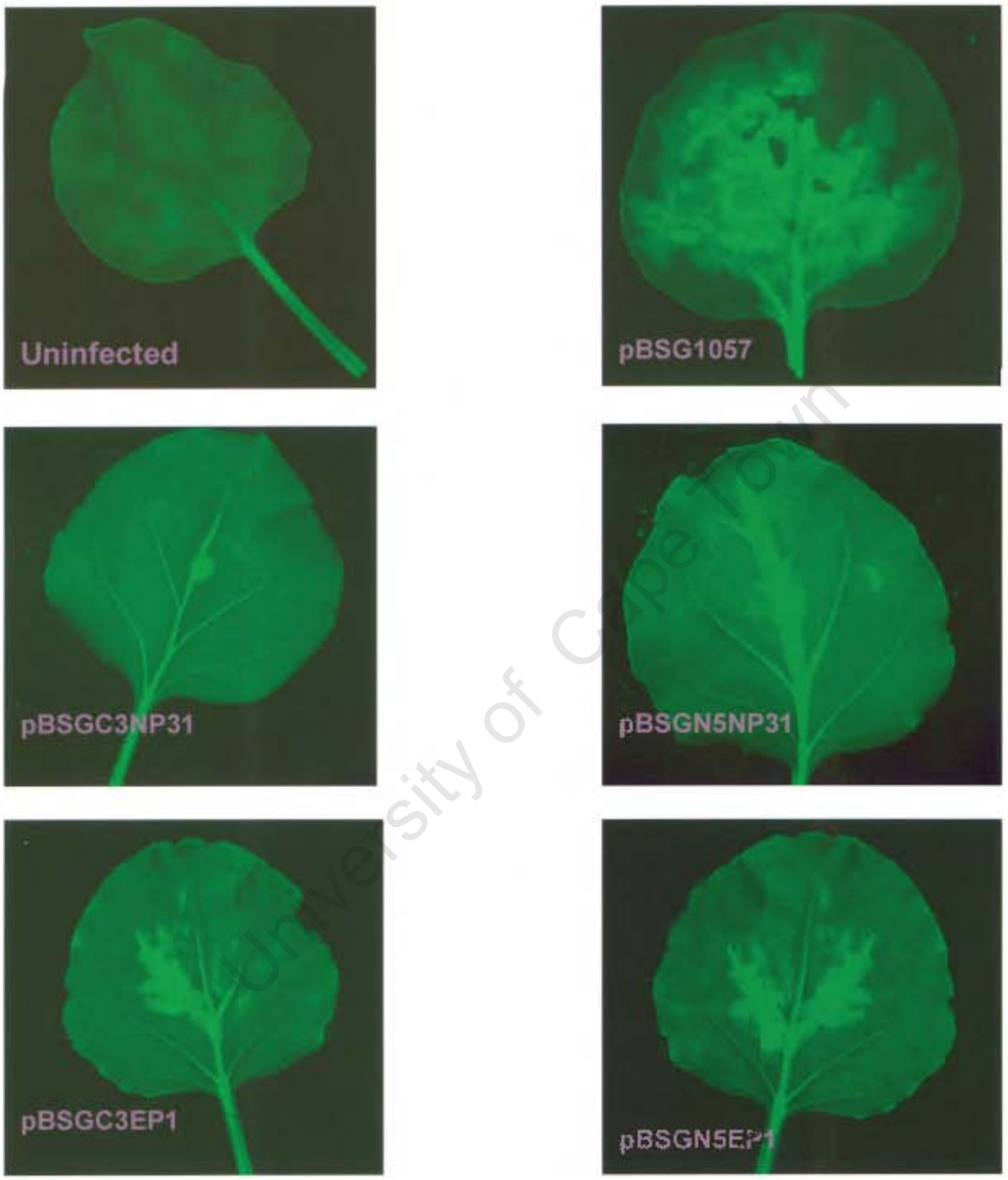


Figure 4.7 Pictures showing typical patterns of spread of GFP through leaves inoculated with pBSGN5NP31, pBSGC3NP31, pBSGN5EP1 and pBSGC3EP1. Pictures were taken under UV light in a Syngene™ UV box.

than the control (pBSG1057) infections. The infection also remained confined to the inoculated leaf and the homogenates of the inoculated leaves were not infectious after repeated attempts to passage and bulk up the infection.

4.3.4 RNA Extraction and RT-PCR

RNA was extracted from the inoculated leaves of the infected plants as well as from adjacent top leaves at the apical meristem. These RNA samples were subjected to RT-PCR to test for the presence of tRNA of the coat protein fusion genes. The results in figure 4.9 show that the coat protein fusion genes were detectable in all of the inoculated leaves but not in the top leaves.

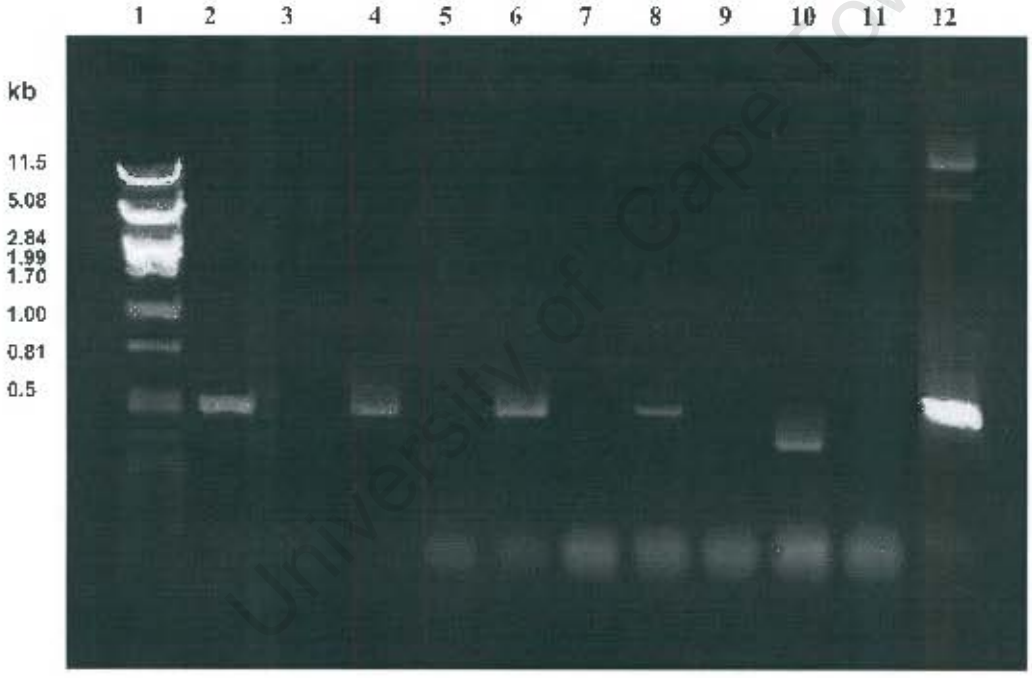


Figure 4.8: Results of RT-PCR of RNA extracted from leaves infected with pBSGN5NP31, pBSGC3NP31, pBSGN5EP1 and pBSGC3EP1. Lane 1, λ/ Pst 1 DNA marker; Lane 2, pBSGN5NP31 inoculated leaf; Lane 3, pBSGN5NP31 plant top leaf; Lane 4, pBSGC3NP31 inoculated leaf; Lane 5 pBSGC3NP31 plant top leaf; Lane 6, pBSGN5EP1 inoculated leaf; Lane 7 pBSGN5EP1 plant top leaf; Lane 8, pBSGC3EP1 inoculated leaf; Lane 9, pBSGC3EP1 plant top leaf and Lane 10, pBSG1057 with gfp primers; Lane 11, uninfected plant with all primers and Lane 12, pBSGN5NP31 plasmid DNA.

4.3.5 SDS PAGE and Western Blotting

Protein extracted from the infected leaves was analysed by SDS PAGE. Figure 4.10 shows a SDS PAGE of the extract of leaves inoculated with pBSGC3NP31, pBSGN5NP31, pBSGC3EP1 and pBSGN5EP1.

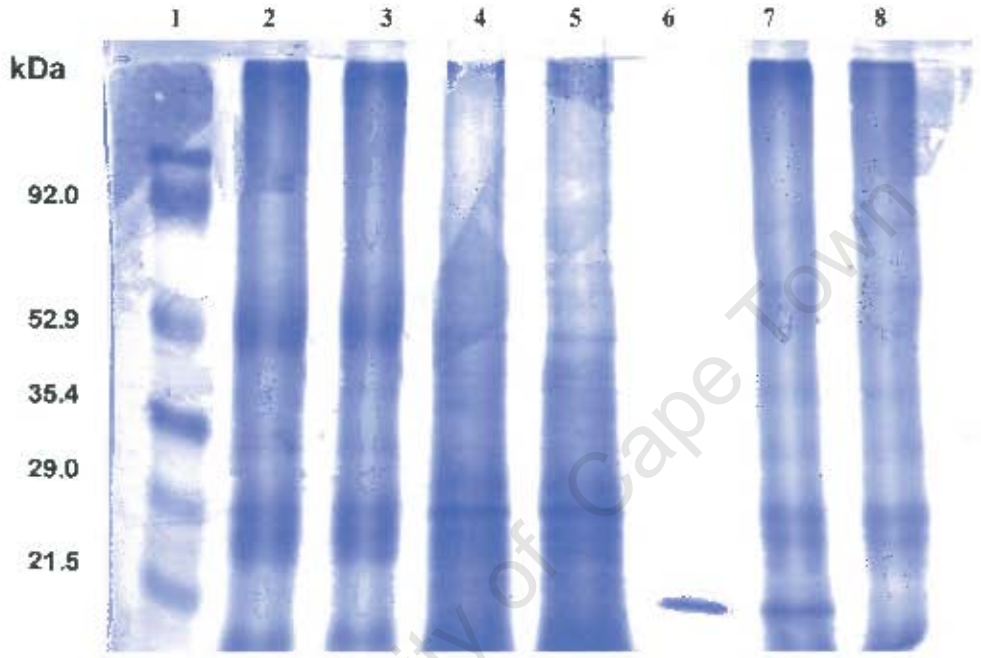


Figure 4.9: SDS-PAGE of plant extract of leaves infected with pBSGN5NP31, pBSGC3NP31, pBSGN5EP1 and pBSGC3EP1. Lane 1, BIORAD low molecular weight protein marker; Lane 2, pBSGN5NP31; Lane 3, pBSGC3NP31; Lane 4, pBSGN5EP1; Lane 5. pBSGC3NP31; Lane 6, pure TMV-CP; Lane 7, pBSG1057 and Lane 8, healthy plant.

GFP was visible in all the inoculated leaves and the presence of the GFP and its position after migration by electrophoresis was confirmed by western blot probed with anti GFP monoclonal antibody (figure 4.11A). The western blot was able to confirm the presence of GFP at high levels in leaves inoculated with the constructs pBSGC3NP31, pBSGN5NP31, pBSGC3EP1 and pBSGN5EP1 as well as in the plant infected with pBSG1057.

The second western blot (figure 4.11B) was probed with an anti TMV CP polyclonal antiserum. The western blot is quite heavily overexposed and it reveals the presence of

high levels of CP in the pBSG1057 infected plant and there is a reaction with the purified rTMV particles loaded in lane 6. In lanes 4 and 5 there is evidence of low levels of expression of rTMV CP in leaves infected with pBSGN5EP1 and pBSGC3EP1.

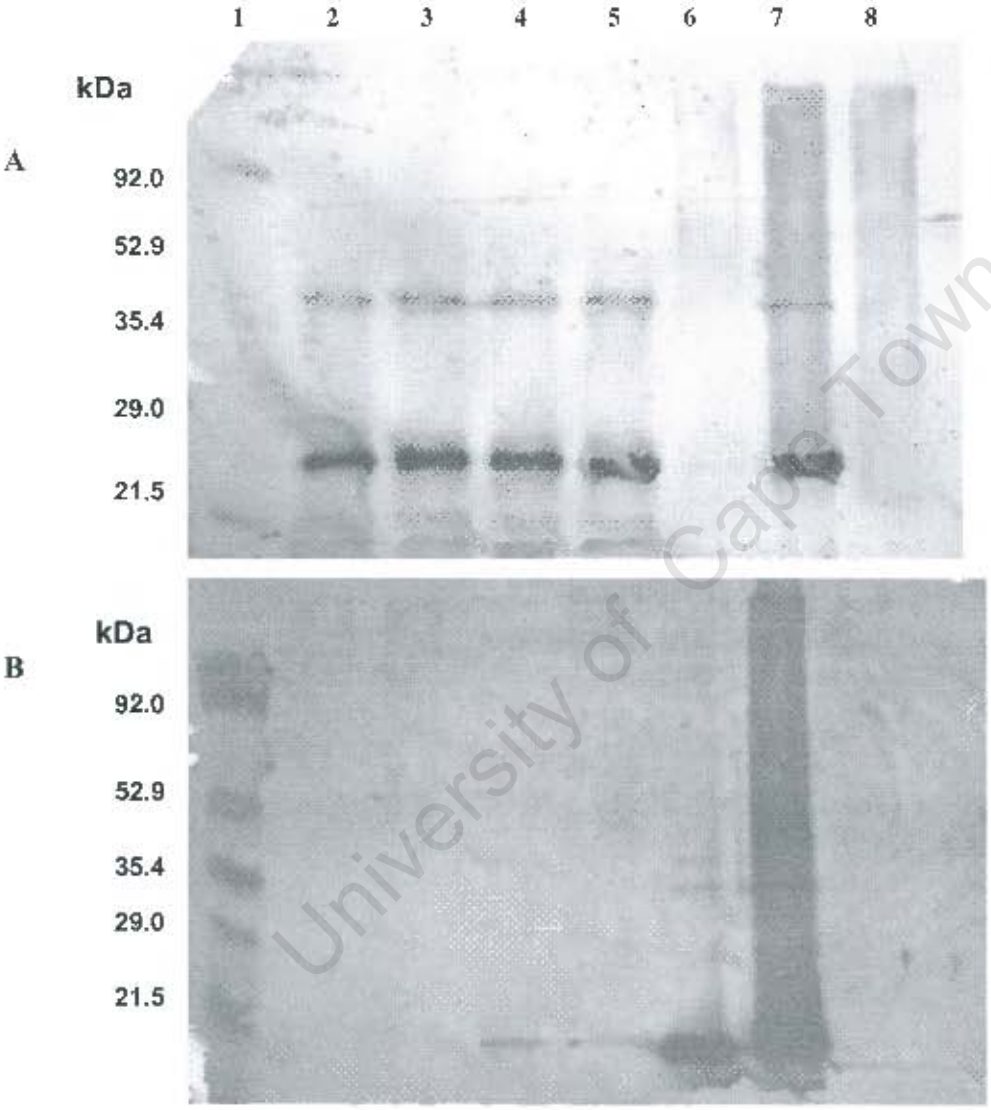


Figure 4.10: Western blots of plant extract from leaves infected with pBSGN5NP31, pBSGC3NP31, pBSGN5EP1 and pBSGC3EP1. A. is probed with anti GFP antiserum while B. is probed with anti TMV antiserum. Loading in both blots is the same. Lane 1, BIORAD low molecular weight protein marker; Lane 2, pBSGN5NP31; Lane 3, pBSGC3NP31; Lane 4, pBSGN5EP1; Lane 5, pBSGC3NP31; Lane 6, pure TMV-CP; Lane 7, pBSG1057 and Lane 8, healthy plant.

4.4 CONCLUSION

Two regions of the HIV-1 subtype C Nef core region were cloned into pBSG1057 as coat protein fusions on both the N and the C termini. Oligonucleotides encoding the immunodominant helical region of the Nef core H1 containing the mapped CTL epitope NP31:YKAAFDLSHFLKEKG and a smaller epitope EP1:EEPEVGFPV, were placed on the C and N termini in frame with the coat protein gene to be translated as a fusion. The use of intermediate vectors in the form of pUCN5 and pUCC3 enabled easier manipulation of the DNA since pBSG1057 is a large vector with a low copy number in bacteria. The intermediate vectors also contain the M13 forward and reverse binding regions where these primers can be used for sequencing purposes. PCR and DNA sequencing were used to confirm the successful construction of the vectors pUCC3NP31, pUCN5NP31, pUCC3EP1 and pUCN5EP1. The CP fusion genes in these vectors were excised using *Sal* 1 and *Pst* 1 restriction digests and placed into the pBSG1057 backbone that had been digested with the same enzymes (see figure 4.2).The presence of the epitope fusion genes in the vectors pBSGN5NP31, pBSGC3NP31, pBSGN5EP1 and pBSGC3EP1 was confirmed by PCR (figure 4.6).

The constructs were all transcribed into infectious RNA (Figure 4.7). This RNA was used to infect young plants and the plants were monitored for the presence of infection. The initial signs of infection were seen after 7 days in plants inoculated with the NP31 fusion constructs and in those inoculated with the EP1 fusion constructs initial spots appeared after 5 days. The highly attenuated state of the infection was particularly evident in the way that the infection spread. In the case of the NP31 fusions, after the appearance of an initial fluorescent spot at a single focus on each leaf, the area of fluorescence enlarged very slightly over the next few days until it reached a vein. The fluorescence then appeared to spread along this vein all the way to the bottom of the leaf and into the stem (figure 4.8). A similar pattern of spread was seen in leaves infected with the EP1 fusion constructs. In both cases, it is notable that there was no difference in the infection between the N-terminal and the C-terminal fusions. In both cases, the infection was

limited to the inoculated leaf and no fluorescence was seen beyond the junction of the leaf stem to stalk of the plant. No rTMV CP RNA was detectable beyond the inoculated leaf either (Figure 4.9). This is evidence of the infectious nucleic acid's inability to execute long distance movement. As a bioassay for the production of virus particles in vivo, the infectivity of homogenates of inoculated leaves showing fluorescence was tested on fresh young plants. The infectivity should roughly estimate the amount of assembled virus particles, since naked genomic RNAs are more RNase-sensitive and less infectious than virus particles (Saito *et al.*, 1990). None of the homogenates tested were infectious. The reason for this becomes clear upon analysis of the plant by SDS PAGE and western blot. In figures 4.10 and 4.11B, there is evidence of poor accumulation of CP. In particular, both pBSGN5NP31 and pBSGC3NP31 accumulate rTMV CP at levels undetectable by western blot. In the case of pBSGN5EP1 and pBSGC3EP1 there is also poor accumulation of CP to levels detectable by western blot but at levels several much reduced relative to those seen in pBSG1057 infected plants. There appeared to be a little more rTMV-CP pBSGN5EP1 inoculated plant than in the pBSGC3EP1 inoculated plant. Such poor accumulation of CP has implications for long distance movement which requires virus assembly (Dawson *et al.*, 1988). The cell to cell movement of infectious RNA requires only the movement protein and that type of movement is evident in the enlargement of the initial fluorescent spots. This mode of spread alone is however not sufficient to cover even the inoculated leaf. Martens *et al.* (2001) showed that the expression of GFP targeted to the ER reflected the sink-source transition in leaves. In this case, the movement of GFP is restricted to cell to cell movement and is therefore also restricted to the cytoplasmic sleeve. This is reflected in the patterns of spread of the GFP in rTMV inoculated plants seen in figure 4.8, which reflects a directed spread of GFP toward the midribs, petioles and stems. Expression of ER-targeted GFP was shown to be restricted to companion cells, but free GFP was trafficked across the pore/plasmodesma contacts, approached the assimilate stream and was transported source-to-sink (Imlau *et al.*, 1999). It is likely that this was also the case in these experiments. It is also likely that macromolecular trafficking of RNA occurred from companion cells to sieve elements across the sieve/pore plasmodesmata contacts typical of this cell pair. This is the method by which assembled virus effects long distance spread.

The reasons for such poor accumulation of coat protein particles are not immediately clear but several inferences can be made based on these results and those of similar work carried out by researchers at LSB Corporation. Several factors may play a role. Not least is the fact that the vector used is designed primarily for use in expression of free proteins. To optimise expression of free protein, the coat protein gene used in construction of this vector is that of the TMV U5 (TMGMV) strain (Shivprasad, 1999) which has a milder symptomology and accumulates much lower levels of coat protein than the U1 TMV CP. Indeed several researchers have found that CP genes from other viruses are more accommodating terminal fusions. For example the protein-protein interactions in AlMV, allows the formation of particles of various shapes such as spherical, ellipsoidal and baciliform depending on the size of RNA to be encapsidated (Shaw, 1996). The AlMV CP has been expressed from a TMV based vector for easier manipulation and this has enabled the expression of a variety of peptides including the entire tat gene as epitope fusions (Belanger *et al.,* 2000). Use of the AlMV has enabled the number of amino acids that can be expressed as a terminal fusion to increase from 25 with TMV-CP (Turpen *et al*, 1995) to 38 (Belanger *et al*, 2000)

In a study by Palmer, 18 different neutralizing and CTL epitopes from human pathogenic papillomaviruses were expressed as N-terminal and 60s loop fusions to the TMV coat protein. In the study it was found that N-terminal display of some epitopes resulted in either no accumulation of coat protein, poor accumulation or high accumulation with very low solubility and assembly capability. The factors determining which epitopes will express well have not been conclusively determined but epitopes with constrained structures appear more likely to interfere with expression of coat protein and solubility and consequently, with infectivity. In another example expression of the 19 amino acid loop sequence from domain 4 of the *Baccilus anthracis* protective antigen was unsuccessfully attempted as an N-terminal fusion of the TMV CP. The epitope was rescued by insertion at the 60s loop position where the structure could be displayed in its native form. The NP31 epitope is generated from the highly conserved and constrained helical H1 domain of the HIV-1 Nef core region and it is possible that given this context

and the already poor accumulation of the TMGMV CP to which it was fused, the protein did not accumulate to any significant levels. It is unlikely however that the epitope fusion gene disrupted expression at a transcriptional level as rTMV mRNA was detectable in extracted total RNA from the leaf. A more likely explanation is that the fused epitope caused irregular folding of the protein causing it to be ubiquitinated and targeted to the 26S proteosome for reprocessing. However, this process alone cannot account for the almost total disappearance of rTMV CP from the examined plant extract as it is known that other functional viral proteins such as the movement protein are able to carry out their normal function despite being subject to this process (Gillespie *et al*, 2001). An alternative explanation is that the epitope fusion caused a drastic reduction in solubility. There are several reports indicating that CP solubility and thus the ability to assemble into virus particles can be affected by terminal or internal additions of peptides (Bendahmane *et al.*, 1999). Because the analysis of plant extract is restricted to that which remains soluble it is likely that the insoluble CP fusion products were retained in the insoluble material that was centrifuged down in the preparation of the plant extract. This would explain the presence of increased amounts of CP in the plants infected with the EP1 fusion products. The much smaller epitope is likely to have affected the solubility of the CP particles to a lesser degree leaving more such particles available. This may have had implications on the movement of the infectious RNA as it is possible that with low quantities of soluble TMV there may have been formation of some RNA-CP aggregates such as helical aggregates short rodlets of TMV containing encapsidated RNA. These structures have been described in transgenic tobacco expressing relatively low levels of the TMV CP (Beachy, 1990). Such aggregates would possibly be loaded into the phloem and allow increased short distance spread of the infectious RNA through the loading veins of the leaf but are unlikely to be robust enough to withstand the plant's vascular environment long enough to effect systemic spread of the infection. This would also explain the increased levels of local spread of GFP expression in leaves inoculated with EP1 fusion constructs relative to those inoculated with NP31 fusion constructs (figure 4.8). The requirement for assembled rTMV remains as this form elicits much higher antibody responses than the disassembled form (Hwang *et al*, 1994). Purification

or concentration of the disassembled CP by filtration for possible immunogenicity tests would be expensive and likely have limited success making it unviable.

Much is still to be uncovered in relation to how best effect expression of epitopes as CP fusions. Several considerations may be useful for future investigators to make. Perhaps the most important is that an appropriate vector with high levels of CP accumulation should probably be used. TMGMV CP is useful in vectors expressing free proteins but may be a hinderence in epitope fusion expression. TMV U1 CP has successfully been used to express a large number of different epitopes. Alternatively, a variety of other CP genes might be used in expression of epitopes. The flexibility of AlMV and potexviruses make them worthwhile options to explore as epitope display systems. Secondly, constrained epitope structures may be problematic to express as terminal CP fusions. Loosely structured sequences might be used with greater success. Heath *et al* (2002) successfully used the same vector to express a 24 amino acid multiepitope sequence constructed by joining end to end 3 short loosely structured epitope sequences. In cases where there is a need to express a particular highly structured epitope, one of two strategies might be successfully amployed. By using a leaky stop codon it is possible to express the construct so that only a proportion of the CP subunits are decorated with the epitope (Awram *et al* 2002). An alternative site of insertion of epitopes such as the 60s loop of the TMV CP may be used. Both methods seem to successfully overcome the problems of expression and solubility associated with decoration of the CP subunit with large epitopes.

The recent results on immunogenicity studies of chimeric virus particles produced in plants suggest that this is going to be a significant source of cheap and effective antigens and vaccine reagents in the near future. Epitope vaccines have not yet escaped the general problem facing subunit vaccines where B cells are stimulated without a corresponding T cell response (Awram *et al*, 2002, Heath *et al* 2002). This problem can be overcome by insertion of the appropriate T-cell antigenic peptides or by use of an adjuvant to stimulate the immune system (Awram *et al*, 2002). This is encouraging news for a continent reeling from the effects of the worst pandemic of the 21st century.

CHAPTER 5

CONCLUSION

The seriousness of the health challenge that HIV/AIDS presents is unprecedented. However, given the advent of recombinant technologies and the resulting possibilities for the use of subunit vaccines, there is hope that rising to the HIV/AIDS challenge is not beyond us. For developing countries, the increase in popularity of plants as a bioreactor of choice for the production of vaccines and other protein products is timely. The need for cheap vaccines and the advantages plants can offer in this and other respects are reviewed in chapter 1 in relation to HIV/AIDS. This need is not limited to HIV/AIDS, however, as although effective vaccines exist for diphtheria, measles, pertussis (whooping cough), polio, tetanus and tuberculosis, these diseases still result in the death of more than 2 million children annually (Awram *et al.*, 2002).

Much progress has been made in the development of vaccines for HIV/AIDS. As work continues on the development of a completely protective neutralizing antibody response in the face of significant difficulties, the development of non-sterilizing and therapeutic immune protective measures is also going forward. The HIV-1 *nef* is an accessory gene that is likely to feature prominently in this regard due to its pivotal pathogenic functions and richness in epitopes of all kinds.

The field of plant-derived vaccines is still in its formative stages, but a great deal is already known about what is likely to give the optimal results. The most successful plant-derived vaccines appear to be those where the greatest yield has been achieved. As a consequence, the importance of achieving high levels of protein expression cannot be overstated. Detailed investigations of the expression of HIV-1 proteins in plants are therefore necessary initial steps in the process of the development of plant-based HIV vaccines. The two methods known to produce the highest yield are the chloroplast transformation and virus-based expression systems. The prokaryotic nature of chloroplasts means virus-based expression systems, which use eukaryotic plant cellular

apparatus, may offer a superior product in terms of post-translational modification of proteins. High protein yields have been generated using TMV-based vectors expressing heterologous genes as free proteins and as fusions.

There is an ever-increasing amount of reagents available for research into HIV. Unfortunately, a disproportionately large number of these reagents are specific to HIV-1 subtypes B and E that are more prevalent in developed countries where the epidemic is more under control. This was underscored by the lack of a commercially available antiserum raised against HIV-1 subtype C Nef. The *E. coli*-based pProEX-HT expression system provided a rapid means of developing an immunoassay that would rely on cross reactivity of Nef-specific antibodies between the various subtypes. The production of His(6)-Nef also made available a reagent that could be used to generate a HIV-1 subtype C Nef-specific antiserum in the event that such a reagent is required for future use. Most interestingly, in a study run concurrently to mine, Finzi *et al.*, (2003) further refined the methods of expressing Nef in *E. coli* by developing a two-step purification process based on the heparin binding ability of Nef and IMAC. This process produces the purified protein in its native configuration. Bacterial expression is likely to remain the most efficient way of rapidly producing heterologous protein reagents on a small scale.

A qualitative indirect ELISA assay was successfully developed for the detection of Nef in *Nicotiana benthamiana* using His(6)-Nef. The best ELISA-based quantitative method would probably have been a sandwich ELISA, which is known for its precision and reproducibility. This method, however, appears to be unpopular probably in part due to its requirement for labeling of antisera specific for each antigen. Western blotting and dot blots, are by far the most popular means of quantitation of heterologous proteins in plants. The relatively low amounts of antiserum required, its reasonable accuracy and in particular the ability to resolve proteins prior to detection made western blotting the ideal method for this study.

Expression of Nef in *Nicotiana benthamiana* as a free protein produced modest results in terms of protein yield (Chapter 3). The levels of Nef expressed in *benthamiana* using

pBSG1057 were even lower than those seen in transgenic plants, although to be fair, I was unable to find an example of a transgenic plant expressing HIV-1 Nef. Nonetheless, accumulation of protein to levels of less than 0.007% of total soluble protein is far from economically viable and would certainly present problems in purification. While filter concentrating the plant extract enabled visual detection of the protein by SDS-PAGE and western blot, the membrane filter devices used are expensive and would not be a viable option on a scaled-up process. A pBSG1057 derivative vector containing a histidine tag has been developed in the laboratory and may be a useful option in the case of persistent low yields.

Expression of Nef as a free protein enabled me to asses the importance of codon optimization in plant protein expression. This aspect of protein expression has been the subject of an ever-increasing number of studies. The *nef* gene was re-synthesized to reflect a plant codon bias based on the codon usage of *Nicotiana benthamiana* and inserted into the TMV vector. Although expression levels remained low, comparisons of levels of protein accumulation in plants expressing the codon optimized gene against that of plants expressing the wild type gene showed that codon optimization resulted in at least a two-fold increase in expression. While the exact mechanisms by which this is achieved are yet to be elucidated, there is a definite case for using codon optimized genes for expression of proteins using TMV. An additional point of interest would be to examine the expression of the gene optimized for TMV codon usage. The ability of TMV to generate significant amounts of some viral products with genes that have a unique and TMV specific codon usage indeed suggests that this is an option well worth exploring.

The recent application of molecular biology to characterize the antigenic determinants of pathogens has resulted in rapid progress in the development of new vaccines using epitope presentation systems. The coat protein of TMV was among the first plant viruses proteins to be used as a carrier molecule for antigenic epitopes. The abundance of well characterized CTL epitopes in Nef means that in all likelihood, successful vaccine candidates for HIV-1 that use this approach will carry some Nef epitopes if they aim to raise a CTL response. Epitope decorated TMV particles have the advantage of being

large particulate antigens which may alleviate the need for an adjuvant. This has usually meant that the responses generated from such particles have been largely B-cell responses even where CTL epitopes have been used. For this reason we can be hopeful that some sort of protective immunity can be generated using antigens from the V3-loop of Env and that when the appropriate response is generated using CTL epitopes it will be a comprehensive and broad immune response. The epitope display system is particularly useful in that several antigenic determinants, even from different pathogens, can be presented on a single construct. The fact that I was unable to produce a chimeric CP that assembled into a decorated virus like particle points to some important considerations that need to be made for the successful use of this system, which have been pointed out already. Most importantly though, the large numbers of different products that have been produced and tested using this approach suggest that vaccines produced this way will soon become a marketable product with clear competitive advantages over currently used vaccines.

While there have been some successful results from studies carried out already, the need for optimization of protein production levels still persists. There is still much scope for improvement of expression levels of free proteins and translational fusions. As pointed out earlier, codon usage is just one of the factors that will influence the expression of free proteins in plants. Other improvements in levels of foreign protein expression, genetic stability and infection phenotypes have been made through the inclusion of extra promoter elements to drive foreign gene expression and the creation of hybrid tobamovirus genomes. In particular, the use of the AlMV coat protein as an epitope carrier molecule is full of possibilities. The very exciting advances that have been made in the development of the PCR based technique of DNA shuffling means that attempts to improve vector efficiency are no longer limited by their reliance on available genetic diversity in virus isolates. Through this process researchers can now seek to evolve optimal viral components and compensate for deficiencies present in the expression vectors. The convergence of transgenic and virus based technologies is particularly useful as it addresses the most expensive aspect of production using virus based methods. The need for generating RNA transcripts is eliminated and costs are further reduced.

There is little doubt that the development of cheap plant based vaccine candidates ready to go into clinical trials is imminent.

APPENDIX A

STANDARD METHODS

STANDARD METHODS

1. BACTERIAL MEDIA, ANTIBIOTICS AND BACTERIAL STRAINS

All bacterial cultures were grown in Luria-Bertani (LB) medium (5g bacto-tryptone, 5g yeast extract, 10g NaCl, 950ml H2O, pH 7.4 adjusted with NaOH, made up to 1L and autoclaved) or Luria agar (LA) plates (Luria Bertani medium with 15g agar). Ampicillin was used at 50μg/ml in LB and 100μg/ml in LA.

Unless otherwise stated all DNA was transformed into and isolated from E. coli strain DH5α.

2. DNA ISOLATION PROCEDURES

Mini-prep DNA was isolated from 5ml cultures grown overnight at 37 C with shaking. Mini-preps were processed by the alkaline hydrolysis method of Sambrooke *et al.*, (1998) using reagents and materials from the Roche® Hydrobond™ plasmid DNA extraction kit.

Maxi-prep DNA was isolated from 50ml cultures grown overnight at 37 C with shaking. Maxi-prep DNA was processed by the alkaline hydrolysis method of Sambrooke *et al.*, (1998) using reagents and materials from the Qiagen Midi plasmid DNA extraction kit.

3. PCR

PCR amplification reactions contained 1pmol of each primer and 8.2ng of template DNA in a total reaction volume of 50μl. $MgCl_2$ was added to a final concentration of 1.25mM and dNTPs were added to a final concentration of 25mM. Roche® PCR buffer and a total of 5 units of Roche® Supertherm DNA polymerase were used.

Reaction mixtures were subjected to an initial denaturatuon step of 94°C for 5 minutes. The reaction mixtres were then subjected to 25 cycles of amplification with a denaturation step of 94 °C for 30 seconds, an annealing step of 55°C for 30 seconds and

an elongation step of 72°C for 30 seconds. DNA amplifications were controlled by a GeneAmp®PCR System 9700 thermocycler. After amplification, samples were analysed by agarose gel electrophoresis.

4. RESTRICTION ENZYME ANALYSIS OF DNA

DNA was routinely digested by the required enzyme under the conditions stated by the supplier (Roche). Two units of enzyme was used per µg of DNA digested for 1.5 hours at 37 C

5. GEL ELECTROPHORESIS OF RNA AND DNA

Gel electrophoresis of RNA and DNA was carried out on 0.8% agarose gels in 1xTBE and 0.5µg/ml ethidium bromide as indicated in Sambrook et al. (1989). For all RNA gels all reagents were prepared RNase free. DNA and RNA were sized against λ-DNA digested with *Pst 1* to give 29 fragments ranging from 15bp to 11497bp.

6. GEL PURIFICATION OF DNA

DNA from a PCR reaction mixture or endonuclease digest was loaded onto a 0.8% TBE agarose gel with 0.5µg/ml ethidium bromide for electrophoresis. The band of interest was visualised on a UV light box and excised using an ethanol-cleaned scalpel. The excised agarose gel piece was placed in a sterile 1.5ml microcentrifuge tube. The DNA was extracted from the agarose gel using reagents from and the protocol outlined in the Roche® High-Pure™ PCR Cleanup kit.

7. LIGATION

Ligations were routinely performed in 10µl with 2 units T4 DNA ligase (Roche) at 4 C overnight. Unless otherwise stated, the vector:insert ratio for small inserts of less than 500 base pairs was 1:8 with the DNA concentration not exceeding 800ng. For larger inserts a higher ratio was used. Ligation of the PCR product with pGEM®-T Easy was performed using reagents provided by the Promega® pGEM®-T Easy kit and following the protocol recommended by the manufacturer. A total of 3µl of the PCR product was used in the standard ligation reaction as per the manufacturer's recommendation.

8. TRANSFORMATION

E. coli cells were made competent for transformation and transformation was carried out using the method of Chung and Miller (1988). Transformations using 5µl of the ligation reactions were conducted using DH5α high efficiency competent cells. Ligation mix and competent cells were mixed in a microfuge tube and chilled on ice for 20 minutes. The cells were then heat shocked by incubating at 42 C for 30 seconds. Thereafter, 600µl of LB was added and the transformation mix was incubated at 37 C for 1 hour with shaking. The transformation mix was plated and selection of transformed cells was done on LB plates with ampicillin. The plasmid pUC19 was used as a standard to check the transformation efficiency of the competent cells used.

9. SEQUENCING

Miniprep DNA was used for sequencing reactions. Purified plasmid DNA was sequenced at the Department of Molecular and Cell Biology's Sequencing Service by a modification of the dideoxy chain termination method of sanger et al. (1977) for dsDNA templates. Cycle sequencing reactions, with universal –80 forward (5'-CGCCAGGGTTTTCCCAGTCACGAC-3'; Pharmacia Biotech) and –80 reverse (5'-GAGCGGATAACAATTTCACACAGG-3'; MWG Biotech) fluorescent-labelled Cy5-Far Red primers was done using the Thermosequence™ cycle sequencing kit (Amersham Life Science) and run on an ALF-Express™ DNA Sequencer version AMV2.0 (Pharmacia Biotech). Labelling and termination reactions were carried out in the presence of 7-Deaza dGTP. Analysis of generated sequences was done using DNAMAN for Windows version 2.6 (Lynon Biosoft, ©1994-1996)

10. RNA EXTRACTION

100mg of leaf material from an inoculated plant was homogenised in liquid. Whilst the leaves were still frozen, 1ml TRIZOL (Biorad) was added to the leaves and incubated for 5 minutes at room temperature. The leaf suspension was centrifuged at 12000g in a microcentrifuge for 10 minutes at 4°C and the supernatant fraction removed and placed in a new tube. 200µl of chloroform was added to the supernatant and shaken for 3 minutes

at room temperature. The tubes were then centrifuged at 12000g for 15 minutes at 4°C. The supernatant was removed and added to 0.5ml of isopropanol and left for 10 minutes at room temperature. The tubes were centrifuged at 12000g for 10 minutes at 4°C and the supernatant discarded. The pellet was washed with 1ml 75% ethanol and centrifuged at 7500g for 5 minutes at 4°C. The ethanol was discarded and the RNA pellet was re-dissolved in 50µl DEPC treated water

11. REVERSE TRANSCRIPTION AND PCR AMPLIFICATION OF cDNA

PCR amplification reactions contained 1pmol of each primer and 1µl of template RNA in a total reaction volume of 50µl and dNTPs were added to a final concentration of 25mM. Primers were added to a final concentration of 0.3µM. DMSO and DTT solutions were added to final concentrations of 7% and 5mM respectively. 20 units of Rnase inhibitor were also added to every reaction tube. Finally RT-PCR buffer and the C. therm. Polymerase buffer mixture were added. The reaction mixture was equilibrated at 60°C and incubated for 30 minutes to allow the reverse transcription reaction. Amplification began with a 2 minute denaturation at 94°C. This was followed by 10 steps of 30 seconds at 94°C, 30 seconds at 65°C and 1 minute at 72°C. The final amplification step consists of 25 cycles of 30 seconds at 94°C, 30 seconds at 65°C and 1 minute at 72°C with a 5 second elongation for each cycle. The results were analysed by gel electrophoresis

12. SDS-PAGE

SDS-PAGE was performed essentially as described by Laemmli (1970). Gel slabs (1.5mm thick) comprising a 15% resolving gel and a 4% stacking gel were poured in a Hoefer SE-600 vertical slab gel apparatus (Hoefer Scientific Instruments). Samples to be electrophoresed were disrupted by mixing them with an equal volume of protein loading buffer (10% [w/v] SDS, 15% 2-mercaptoethanol, 15% glycerol, 0.01% bromophenol blue, 0.125M Tris-HCl pH6.8) and heated to 100 C for 10 minutes. Electrophoresis was carried out with a constant current of 30mA until the tracking dye front reached the gel base. 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 and de-stained with repeated changes of the same mixture without the PAGE blue 83.

13. BRADFORD ASSAY

5µl of total protein extract was added to 95µl water and then added to 900µl of Bradford reagent (Biorad). The solution was gently mixed to avoid bubble formation and the absorbance of the sample was read at 595nm. The measurements were done between 5 and 45 minutes after the addition of the Bradford reagent to the sample. The protein concentration was calculated by comparing the absorbance of the sample with a calibration curve created using BSA samples ranging in concentration from 10-100µg/ml

14. WESTERN BLOT

Protein from SDS-PAGE gels was transferred onto nitrocellulose by a semi-dry blotting method using an electroblotter (Bio-Rad™). Blotting was done at 10V for 30min. The membranes were then blocked for 30min at room temperature in blocking buffer and incubated in sheep polyclonal anti-HIV *nef* antibody (provided by Clive Gray of the National Institute of Virology) diluted 5000 times in blocking buffer overnight at 4°C with shaking. The blots were then washed 4 × 15-20min in blocking buffer with shaking. The blots were then incubated in alkaline phosphatase conjugated, mouse ant sheep/goat IgG (Sigma), diluted in blocking buffer (1:50000) at room temperature with shaking. They were then washed 4 × 15-20min in blocking buffer without the milk. Detection was via colorimetric methods using 100µg/ml NBT and 50µg/ml BCIP, diluted in substrate buffer. Rinsing the blot in dH2O stopped the reaction.

15. INDIRECT ELISA

Dilutions of plant protein extract and controls were made in PBS. These samples were placed in the bottom wells on a Polysorb multi-well ELISA plate and incubated at 4°C overnight. The ELISA plate was given four short washes with PBS with 500ul Tween 20 per litre. Blocking was carried out by adding 200µl of blocking buffer (1% milk powder in PBS with tween) to the wells and incubating at room temperature for an hour. The wells were washed four times with PBS with Tween 20. 100µl sheep polyclonal anti

HIV-1 Nef antiserum diluted in blocking buffer was added and incubated at room temperature for 2hrs. This was followed by 4 washes with 300µl per well of PBS with Tween. 100µl of mouse anti goat/sheep IgG diluted in blocking buffer and incubated at 37°C for an hour. Wells were washed four times with 300µl of PBS with tween. 100µl PNNP was added to each well and the plate was left in the dark for 30 minutes at room temperature. The reaction was stopped with 50µl of 0.5 M H_2SO_4. The absorbances were read at 405nm and printed out for processing.

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